

Fatty acid metabolism in HepG2 cells:
Limitations in the accumulation of
docosahexaenoic acid in cell membranes

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Abstract

The current dietary recommendations for optimal health are designed to increase our intake of two bioactive omega-3 (n-3) fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), abundant naturally in fatty fish such as salmon. Health authorities recommend that the general population consume two to three fatty fish meals per week (1) for optimal health and for the prevention of cardiovascular disease. However, some modern Western societies consume only modest amounts of fish and seafood (2;3). Land based vegetable oils may provide an alternative to meet these needs. Linseed and canola oils are rich in α -linolenic acid (ALA, 18:3n-3) (4). ALA can be converted endogenously to EPA and DHA and suggests that increasing the dietary intake of ALA may increase the conversion and accumulation of DHA in tissues and plasma. However, elevated dietary intakes of ALA in animals and humans results in an increased level of EPA in tissues yet there is little or no change in the level of DHA (5-7). The current consensus is that the synthesis of DHA from ALA in humans is limited yet the mechanisms involved in regulating the accumulation of DHA in tissues are poorly understood.

The reputed rate-limiting enzyme in the conversion of fatty acids is $\Delta 6$ desaturase (D6D). ALA is a substrate for D6D and undergoes a series of desaturation and elongation reactions to yield n-3 long chain polyunsaturated fatty acids (LCPUFA). The final step in the synthesis of DHA from ALA involves translocation of its immediate fatty acid precursor, 24:6n-3 from the endoplasmic reticulum to the peroxisome to be partially β -oxidised to yield DHA. The involvement of multiple enzymes in the desaturation-elongation pathway, and the integration of other

pathways, such as phospholipid biosynthesis, suggests there are various steps that may regulate the accumulation of DHA in cell membranes. This thesis aimed to examine the possible regulatory steps in the conversion of fatty acids to LCPUFA, particularly in the synthesis of DHA from n-3 fatty acid precursors.

The human hepatoma cell line, HepG2, was used as an *in vitro* cell system to examine the accumulation of individual fatty acids and their metabolites in isolation from other competing fatty acid substrates. The accumulation of linoleic acid (LA, 18:2n-6) and ALA in HepG2 cell phospholipids following supplementation with increasing concentrations of each respective fatty acid correlated with that described *in vivo*, as was the accumulation of their conversion products. The accumulation of DHA in cells supplemented with ALA reached a plateau at concentrations above 5 µg/ml and paralleled the accumulation of 24:6n-3 in cell phospholipids, suggesting that the $\Delta 6$ desaturation of 24:6n-3 was prevented by increasing concentrations of ALA, thereby limiting the accumulation of DHA. The accumulation of DHA in cells supplemented with eicosapentaenoic acid (EPA, 20:5n-3) or docosapentaenoic acid (DPA, 22:5n-3) was significantly greater than the level of DHA that accumulated in cells supplemented with ALA. However, regardless of substrate, the level of DHA in cell membranes reached a plateau at substrate concentrations above 5 µg/ml.

This thesis further aimed to examine the effect of fatty acid supplementation on the mRNA expression of D6D in HepG2 cells. The expression and activity of D6D mRNA is subject to nutritional and hormonal regulation. The mRNA expression of D6D in HepG2 cells following supplementation with oleic acid (OA, 18:1n-9), LA, ALA, arachidonic acid (AA, 20:4n-6) or EPA was examined by real time RT PCR.

The expression of D6D mRNA was reduced by up to 50% in cells supplemented with OA, LA, ALA, AA or EPA compared with control cells and suggests that fatty acids modulate the expression of the key enzyme involved in the conversion of fatty acids.

The effect of fatty acid co-supplementation on the fatty acid composition of HepG2 cell phospholipids was also examined in an attempt to gain insights into the role of D6D and the enzymes involved in peroxisomal β -oxidation on the accumulation of DHA from n-3 fatty acid precursors. The reduction in the accumulation of DHA in cells co-supplemented with DPA and docosatetraenoic acid (DTA, 22:4n-6) was greater than in cells co-supplemented with DPA and LA, suggesting that peroxisomal β -oxidation may have a greater role in determining the accumulation of DHA from DPA than the activity of D6D. Further investigation should be directed towards understanding the role that peroxisomal β -oxidation may play in the synthesis of DHA from precursor fatty acids.

The fatty acid composition of cell membranes *in vivo* is a result of several physiological processes including dietary intake, phospholipids biosynthesis and fatty acid conversion as well as catabolic processes. This thesis demonstrates that a greater understanding of the regulation of the conversion of fatty acids will help to define dietary approaches that enhance the synthesis of n-3 LCPUFA from n-3 fatty acid precursors to lead to improved outcomes for health.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Roxanne Portolesi

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LIST OF ABBREVIATIONS

A

1-acyl-GCP	1-acyl- <i>sn</i> -glycero-3-phosphocholine
AA	arachidonic acid
ACC	acetyl CoA carboxylase
ALA	α -linolenic acid
ALD	adrenoleukodystrophy
ANOVA	analysis of variance
ATP	adenosine triphosphate

B

bp	base pair
BSA	bovine serum albumin

C

cDNA	complementary DNA
CPT	carnitine palmitoyl transferase
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CT	threshold cycle

D

d	day
D5D	Δ 5 desaturase
D6D	Δ 6 desaturase
DHA	docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid
DTA	docosatetraenoic acid

E

EDTA	ethylenediaminetetraacetic acid
EFAD	essential fatty acid deficiency
en	energy
EPA	eicosapentaenoic acid

F

FABP	fatty acid binding protein
FAME	fatty acid methyl ester
FAS	fatty acyl-CoA synthetase
FAT	fatty acid transport (protein)
FBS	foetal bovine serum
FFA	free fatty acid

G

GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GLA	γ -linolenic acid
GM	genetically modified

H	
h	hour
L	
LA	linoleic acid
LCPUFA	long chain polyunsaturated fatty acid
LDH	lactate dehydrogenase
I	
INT	tetrazolium salt
M	
min	minute
mRNA	messenger ribonucleic acid
N	
NADPH	nicotinamide adenine dinucleotide phosphate
ND	not detected
NEFA	non-esterified fatty acid
NH&MRC	National Health and Medical Research Council
O	
OA	oleic acid
P	
PBS	phosphate buffered saline
PC	phosphatidyl choline
PCR	polymerase chain reaction
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-activated receptor response element
PUFA	polyunsaturated fatty acid
R	
RCT	randomised control trial
RIPA	radio immuno precipitation assay
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcribed PCR
S	
s	second
SC-26196	2, 2-diphenyl-5-(4-(8)piperazine-1-yl)pentanenitrile
SCD	stearoyl-CoA desaturase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
T	
TCA	tricarboxylic acid
TG	triglyceride
TLC	thin layer chromatography

V	
v	volume
VLCFA	very long chain fatty acid
vs	versus
W	
wt	weight

1 Literature Review

1.1 DIETARY FAT AND FATTY ACIDS

One of the major energy sources in the typical Western diet is fat, accounting for 26-53% total energy (9;10). Fat occurs naturally in foods such as red meat, fish, dairy foods, eggs and oils. Aside from its principal role in providing energy to the body, dietary fat also influences many physiological processes. The fatty acid composition of cell membranes modulates its structure, fluidity and function, including the production of eicosanoids and other secondary messengers (11), receptor function, and enzyme activity (12). Increased cellular fatty acid levels stimulate fatty acid oxidation (both mitochondrial and peroxisomal), inhibit glucose uptake and glycolysis and promote gluconeogenesis (13). The fatty acid composition of cell membranes therefore plays a major role in regulating cell metabolism.

Dietary fat commonly consists of three fatty acids esterified to a glycerol backbone to form a triglyceride. Generally, fatty acids are hydrocarbon chains with a carboxyl group at one end and a methyl group at the other and can be divided into three groups according to their degree of saturation (number of double bonds between carbon atoms). Saturated fatty acids are straight chain fatty acids of variable carbon chain length with no double bonds, monounsaturated fatty acids have one double bond along the fatty acid chain and polyunsaturated fatty acids (PUFA) contain more than one double bond in the fatty acid chain. Fatty acids are systematically named by the number of carbons in the fatty acid chain and the number of double bonds (14). For example, a saturated fatty acid with 16 carbon atoms is systematically named hexadecanoic acid (16:0), although its common name is palmitic acid. Linoleic acid (LA, 18:2n-6) and α -linolenic acid (ALA, 18:3n-3) belong to the omega-6 (n-6) and

omega-3 (n-3) families, respectively. The 'n' nomenclature refers to the position of the double bonds within the carbon chain from the methyl terminus. N-6 fatty acids have their first double bond on the sixth carbon atom from the methyl end, whereas n-3 fatty acids have their first double bond on the third carbon atom.

In 1929, Burr *et al.* (1929) (15) identified LA and ALA as essential for growth and reproduction and in preventing the symptoms of essential fatty acid deficiency (EFAD). These symptoms include eczema and poor growth. ALA is found in plant chloroplast membranes where it is synthesized from LA, which in turn is derived from acetate (16). In plants, ALA is found in leaves, mainly in glycolipids, and as triglycerides in certain seed oils (rapeseed, flaxseed, perilla seed, chia seed), beans (soybeans, navy beans) and nuts (walnuts) (17). LA is a major component of safflower oil. The enzymes responsible for the synthesis of LA and ALA from precursor fatty acids are the $\Delta 12$ and $\Delta 15$ desaturases. Whilst plants express $\Delta 12$ and $\Delta 15$ desaturase, animals, including humans, do not and must consume dietary LA and ALA to prevent symptoms of EFAD. As such, ALA and LA are termed essential fatty acids. The daily dietary intake of ALA in the Western diet is approximately 1.5 g/d compared with the daily intake of LA of 10.8 g (5).

In animals, including humans, ALA and LA are fatty acid precursors to the 20 carbon and 22 carbon PUFA. Arachidonic acid (AA, 20:4n-6) is derived from LA and is the main substrate for a series of enzymes that produce the 2-series eicosanoids, such as thromboxane, prostaglandins, prostacyclins, leukotrienes and lipoxins (18). ALA can be converted endogenously to eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), although EPA and DHA can be consumed directly through the diet. EPA and DHA are abundant naturally in fatty

fish such as salmon. However, minimal quantities of EPA and DHA are consumed compared with LA and ALA. The average intakes of EPA and DHA are 0.056g/d and 0.106g/d, respectively (19).

EPA and DHA are putative antiinflammatory, antiarrhythmic and antiatherogenic agents with potent triglyceride lowering effects, and some activity on plasma cholesterol, particularly in hypercholesterolemic patients (20). EPA negates the proinflammatory and atherogenic effects of lipid mediators derived from AA through its conversion to 3-series eicosanoids (21). The cardiovascular disease prevention conferred by EPA and DHA is attributed to these physiological effects and highlights the role that dietary fatty acids play in maintaining health. Mounting evidence for a positive role of n-3 fatty acids in health has resulted in health authorities recommending an increase in the dietary intake of EPA and DHA. The American Heart Association recommends that the general population consume two to three fatty fish meals per week (equivalent to 0.43-0.57 g/d EPA and DHA) (1) for optimal health.

However, some modern Western societies consume only modest amounts of fish and seafood (2;3). For instance, Australian adults consume 1/6 the quantity of fish and seafood as compared to meat, poultry and game (2;3). The recommended consumption of fish calls for major changes in dietary habits which are unlikely due to public perceptions of possible mercury contamination in seafood, dislike of the taste and/or smell of fish or the perception that seafood is more expensive than meat (22;23). As an alternative, the diet may be enriched with n-3 long chain polyunsaturated fatty acids (LCPUFA) by consuming modified foods high in n-3 LCPUFA or with fish oil supplements, rich in EPA and DHA. These practices

effectively elevate the n-3 LCPUFA status of individuals but rely heavily on declining global fish stocks (24). Exploitation of this natural resource has reduced catch size, both the number of fish and their maturity (24) and is no longer environmentally sustainable. Single-cell oils derived from the fermentation of microorganisms (microalgae or microfungi) are another source of DHA (25) but are expensive to produce in significant quantities (26). Plant oils have the potential to be developed into sustainable and affordable sources of n-3 LCPUFA through the transgenic expression of genes encoding LCPUFA biosynthetic pathways from other organisms (27;28). For the first time in seed oils, DHA has been synthesised from 18 carbon fatty acid precursors in the *Arabidopsis thaliana* plant (28). Public acceptance of the production of oils from genetically modified (GM) plants will be a major challenge for this industry as consumer attitudes towards GM food products are largely negative in many of the developed countries (29). This combination of factors clearly indicates a need for an alternative sustainable source of n-3 LCPUFA to meet the current dietary recommendations for health.

Land based vegetable oils, such as linseed and canola oils, may provide an alternative to meet the n-3 recommendations. Since ALA can be converted to EPA and DHA endogenously, it was thought that increasing the dietary intake of ALA may increase the conversion and accumulation of DHA in tissues and plasma. However, whilst elevated dietary intakes of ALA in animals and humans results in an increased level of EPA in tissues, there is little or no change in the level of DHA (5-7). Direct measurement of fatty acid conversion in humans using labelled ALA supports the conversion of ALA to EPA and docosapentaenoic acid (DPA, 22:5n-3) with limited conversion to DHA (6;30-32) suggesting a complex relationship between ingested ALA and its conversion to DHA.

1.2 HEALTH BENEFITS OF N-3 FATTY ACIDS

Among the n-9, n-6, n-3 and saturated fatty acids, the n-3 fatty acids are associated with improved health outcomes. EPA and DHA have shown particular efficacy in the prevention of cardiovascular disease (33-37) and in infant development (38-43) and are also beneficially implicated in arthritis (44) and diabetes (45-47). Whilst cell culture and animal studies support a preventive action of n-3 LCPUFA in carcinogenesis (48;49), there is divided opinion on the association between n-3 LCPUFA intake and cancer risk in humans. An extensive systematic review of 20 prospective cohort studies concluded that there was no significant association with n-3 fatty acids and the incidence of cancer (50). The role of n-3 LCPUFA in cardiovascular disease and infant development are two areas that have received considerable research.

The association between the dietary intake of n-3 LCPUFA and cardiovascular disease prevention began when Bang and Dyerberg (1980) (51) reported low mortality rates from ischemic heart disease in Inuits consuming a high proportion of fish. Several intervention studies have also shown an improvement in cardiovascular health following n-3 fatty acid supplementation (33-35;52). Bucher *et al.* (2002) (53) performed a meta-analysis of 11 randomised control trials (RCT) that compared dietary or non-dietary (supplements) intake of n-3 LCPUFA (EPA and DHA) with a control diet or placebo in patients with coronary heart disease and found significant protective effects of EPA and DHA on rates of fatal myocardial infarction, sudden death and overall mortality in treatment groups. In contrast, a Cochrane review by Hooper *et al.* (2004) (54) concluded that the evidence to support a role for dietary n-3 fatty acids in cardiovascular disease prevention was inconclusive. The Cochrane review has challenged the current opinion and sparked several responses (55-57) to

refute the conclusions by Hooper *et al.* (2004) (54). One of the major criticisms of the Cochrane review was the inclusion of the follow-up to the Diet and Reinfarction Trial (DART 2) (58), which contributed to the significant heterogeneity between studies included in the analysis. Omitting the DART 2 Study produced similar relative risks for cardiovascular disease reported by Bucher *et al.* (2002) (53). The 20% reduction in all-cause mortality reported in patients with pre-existing coronary heart disease supplemented with 850 mg EPA and DHA in the GISSI Trial (36) is striking evidence for a benefit. There are also sound biochemical theories to explain the health benefits conferred by n-3 LCPUFA in preventing cardiovascular disease including their activity as antiinflammatory, antiatherogenic and antiarrhythmic agents (33;59). A difficulty in systematic reviews is the heterogeneity between studies, including differences in population characteristics, lifestyle, dose and type of n-3 LCPUFA. Overall however, there is evidence to support a role of n-3 fatty acids in cardiovascular health.

Benefits of n-3 LCPUFA have also been described for infants. Nutritional intervention studies in infants have been spurred by reported differences in cognitive development of breast fed infants and their formula fed counter-parts (43), an observation which has been attributed to the fact that human breast milk is a rich source of DHA, whereas standard infant formulas contain no DHA (60-62). Controversy exists regarding the addition of preformed arachidonic acid (AA, 20:4n-6) and DHA, as found in human milk, to infant formulas containing only LA and ALA, for optimal brain development and to ensure the long-term integrity of functional outcomes, such as growth, in infants. A Cochrane review of n-3 and n-6 LCPUFA supplementation in preterm infants, which included 11 RCT assessing the clinical effects of feeding formula supplemented with n-3 and n-6 LCPUFA,

concluded that no long-term benefits were demonstrated for infants receiving LCPUFA and there was no evidence that supplementation of formula with n-3 and n-6 LCPUFA impaired the growth of preterm infants (63). A review of ten RCT designed to test the efficacy and safety of adding either n-3 LCPUFA or a combination of n-3 LCPUFA and AA to formulas for term infants (64) found mixed evidence for support of an effect of dietary n-3 and n-6 LCPUFA on either visual acuity or mental development. The current consensus is that the benefits of DHA supplementation for term infants are smaller than for preterm infants (65). The strongest evidence for good developmental outcomes for infants is breastfeeding for at least six months (65).

1.3 FATTY ACID METABOLISM

Fatty acids are ingested as triglycerides and phospholipids and hydrolysed in the small intestine by pancreatic lipase and other enzymes. Hydrolysis products absorbed by the intestinal mucosa are recombined into triglycerides and phospholipids, which combine with apoproteins to form lipoproteins (66). This process solubilises the fatty acids and permits their transport through the blood to other tissues where the fatty acid can enter various metabolic pathways.

1.3.1 Fatty acid transport

Fatty acids are transported between organs in the blood either as non-esterified fatty acids (NEFA) complexed to albumin or as triglycerides and phospholipids associated with chylomicrons and very low density lipoproteins. Fatty acids associated with lipoproteins can be hydrolysed by two related lipases, hepatic lipase and lipoprotein lipase (67) enabling entry of fatty acids into the cell either by passive diffusion (14;68) or facilitated membrane transport (14;67;69). Non-protein mediated, passive entry of fatty acids involves adsorption to the membrane, protonation, flip-flop

within the membrane and desorption into the cytosolic space (68). Various factors can influence the rate of passive fatty acid movement across cell membranes. These include the affinity of fatty acid binding to sites on albumin (70), the transmembrane pH gradient, the transmembrane gradient of fatty acids, modification of the fatty acid to membrane impermeable derivatives on the trans side of the membrane and the utilisation of the fatty acid for anabolic and catabolic processes (71).

Transport of fatty acids through cell membranes may also be facilitated by proteins, including fatty acid binding proteins, fatty acid translocase, (FAT, also known as CD36) and fatty acid transport proteins. All three proteins are regulated to some degree by members of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors (14;72-74). Inside the cell, fatty acids are bound to cytoplasmic fatty acid binding proteins (FABP) (71;75). It is believed that FABP solubilise and protect fatty acid ligands in aqueous spaces and facilitate their transport in the cytosol (76). There is evidence that FABP may influence fatty acid metabolism through facilitating uptake, transport, sequestration, and/or metabolic targeting of fatty acids (77;78). The discovery of these transport proteins indicates a potential regulatory point of entry for fatty acids into various metabolic pathways. The cellular uptake of fatty acids with their entry into these pathways may be affected by changes in the presence and/or activity of these transport proteins.

1.3.2 Activation and entry of fatty acids into several metabolic pathways

Once inside the cell, fatty acids can enter several metabolic pathways (Figure 1-1). A requisite step before entry into these pathways involves activation of fatty acids by the action of fatty acyl-CoA synthetases (FAS), abundant in microsomes and

mitochondria (75), to form fatty acyl-CoA-thioesters. The FAS enzymes differ from each other with respect to their subcellular locations and their specificities for fatty acids of different chain lengths. The overlapping chain length specificities and tissue distributions mean that most saturated or unsaturated fatty acids in the range of 2-22 carbons can be activated in animal tissues, although at different rates (14). Activated fatty acids are also bound to FABP (67) and serve as a reservoir for intracellular fatty acyl-CoA that can be transported to various subcellular compartments (79). Within these compartments, the activated fatty acids can be substrates for;

- i. β -oxidation; (see Section 1.4)
- ii. elongation and desaturation reactions; or (see Section 1.5)
- iii. assimilation into complex lipids, including triglycerides, cholesterol esters or phospho- and sphingo-lipids (see Section 1.6).

Dietary fatty acid intake, fatty acid conversion and acylation into membrane lipids has a concerted influence on the fatty acid composition of cell membranes and thereby, cellular function.

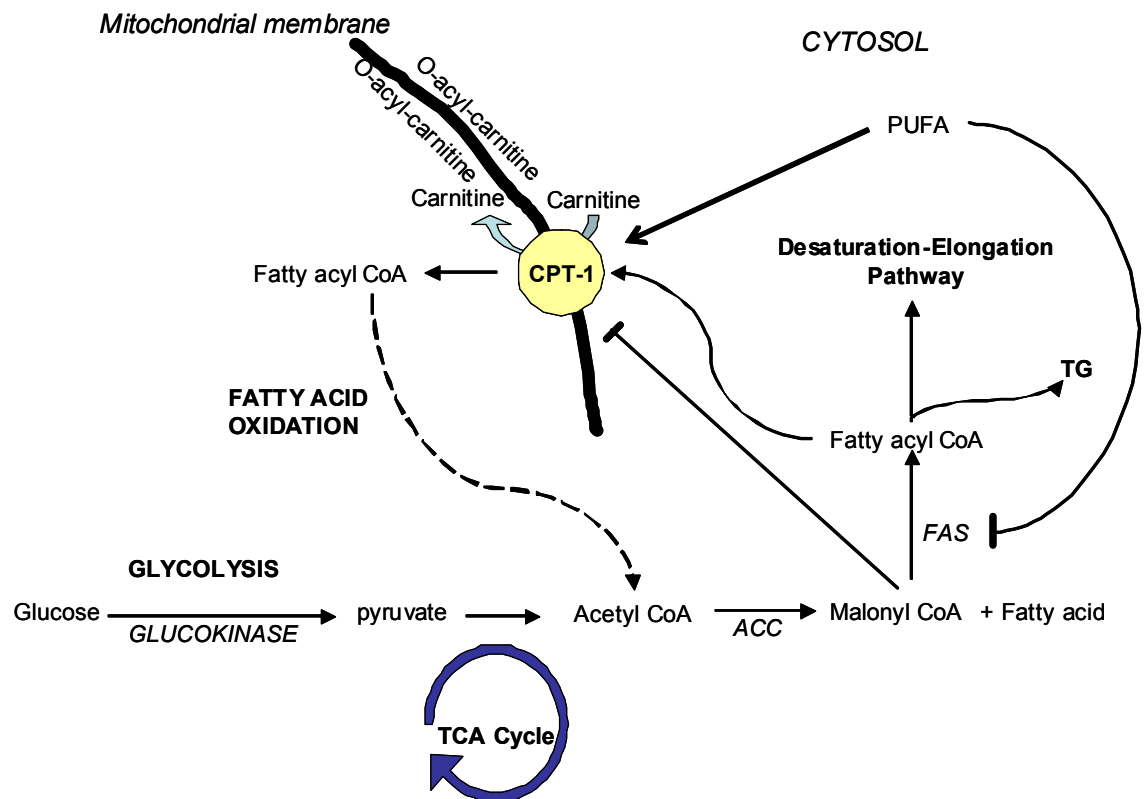


Figure 1-1. Activation of fatty acids in the cytosol and entry into the desaturation-elongation pathway or mitochondrial β -oxidation.

PUFA concomitantly increase the expression of CPT-1 and decrease the expression of FAS, directing fatty acids towards oxidation and away from the desaturation-elongation pathway. Malonyl CoA binds to the N-terminal of CPT-1, inhibiting the transport of fatty acid across the mitochondrial membrane. (*ACC*; Acetyl CoA carboxylase, *FAS*; fatty acyl-CoA synthetase, *CPT-1*; carnitine palmitoyltransferase-1, PUFA; polyunsaturated fatty acid, TG; triglyceride, TCA cycle; tricarboxylic acid cycle).

The expression of genes coding for the enzymes involved in the activation of fatty acids is regulated by fatty acids themselves. Nakamura *et al.* (2000) (80) reported that rats fed a fat-free diet supplemented with 4 weight (wt) % LA reduced hepatic FAS mRNA expression by approximately 50%. Ide *et al.* (2000) (81) report that hepatic FAS mRNA expression was reduced in rats fed a 15% fat diet as either perilla oil (containing ~60% total fatty acids as ALA), safflower oil (~77% total fatty acids as LA), linseed oil (~50% total fatty acids as ALA) and fish oils compared with rats fed palm oil (40-55% total fatty acids as 16:0). The reduced expression of FAS by dietary PUFA suggests that the activation of fatty acids for entry into metabolic pathways is tightly controlled.

De novo fatty acid synthesis involves acetyl CoA carboxylase (ACC) and FAS. The carboxylation of acetyl CoA to malonyl CoA is the first committed step in fatty acid synthesis. The malonyl CoA generated by the action of ACC forms the source of nearly all the carbons for the fatty acyl chain. The second step involves the activation of the fatty acyl CoA by FAS in the presence of NADPH, which results in the esterification of the fatty acid, as described above.

1.4 β -OXIDATION

The entry of fatty acids into catabolic pathways, such as β -oxidation, is also regulated by PUFA. Fatty acids enhance PUFA transport into mitochondria, the major site of fatty acid β -oxidation, via their action on carnitine palmitoyl transferase (CPT). The inner mitochondrial membrane is impermeable to CoA and its derivatives, therefore fatty acyl CoAs formed in the cytosol cannot directly enter the mitochondria for β -oxidation. Entry of activated fatty acids into the mitochondrion is governed by CPT, which transfers the long chain acyl groups from CoA to

carnitine to yield a fatty acyl carnitine. Two isoforms of CPT have been identified (82). CPT-1 is located on the outer mitochondrial membrane, and CPT-2 is on the inner membrane. Peroxisomes and the endoplasmic reticulum also contain CPTs, all of which are inhibited by malonyl CoA (75). PUFA have been shown to regulate the activity of CPT-1, thereby regulating entry into fatty acid oxidation (81;83;84). Ide *et al.* (2000) (81) reports that the activity of CPT-1 is increased by 50% in the liver of rats fed dietary perilla and fish oil, compared to those supplemented with palm or safflower oil. PUFA enhance fatty acid oxidation by co-ordinately increasing the expression of mitochondrial CPT and peroxisomal acyl CoA oxidase, by decreasing the synthesis of malonyl CoA and by decreasing the sensitivity of CPT to malonyl CoA inhibition (83;84). CPT therefore represents another site in the regulation of energy metabolism by PUFA.

Both mitochondria and peroxisomes carry out fatty acid β -oxidation. This cyclic process is similar in both organelles, with each cycle containing dehydrogenation/oxidation, hydration and thiolitic cleavage steps (Figure 1-2). In general, one cycle of β -oxidation shortens an acyl chain by two carbons, releasing one molecule of acetyl-CoA in a process mediated by a sequence of enzymes, each of which is specific for its substrate. However, there are some differences in β -oxidation between the two organelles. In mitochondrial β -oxidation, the preferred substrates are fatty acids with a chain length of less than 20 carbons. These fatty acids enter the organelle by the carnitine transport system and are usually degraded completely to acetyl-CoA via several β -oxidation cycles. Peroxisomal fatty acid β -oxidation is capable of oxidising very long chain fatty acids of 24 carbons. Entry of these substrates does not require carnitine but may involve ATP-binding cassette transporters such as the adrenoleukodystrophy (ALD) protein (85). Peroxisomal β -

oxidation does not proceed to completion but rather only through a few cycles in which the acyl chain is shortened and the shortened fatty acyl CoA returns to the endoplasmic reticulum for assimilation into cell lipids (85).

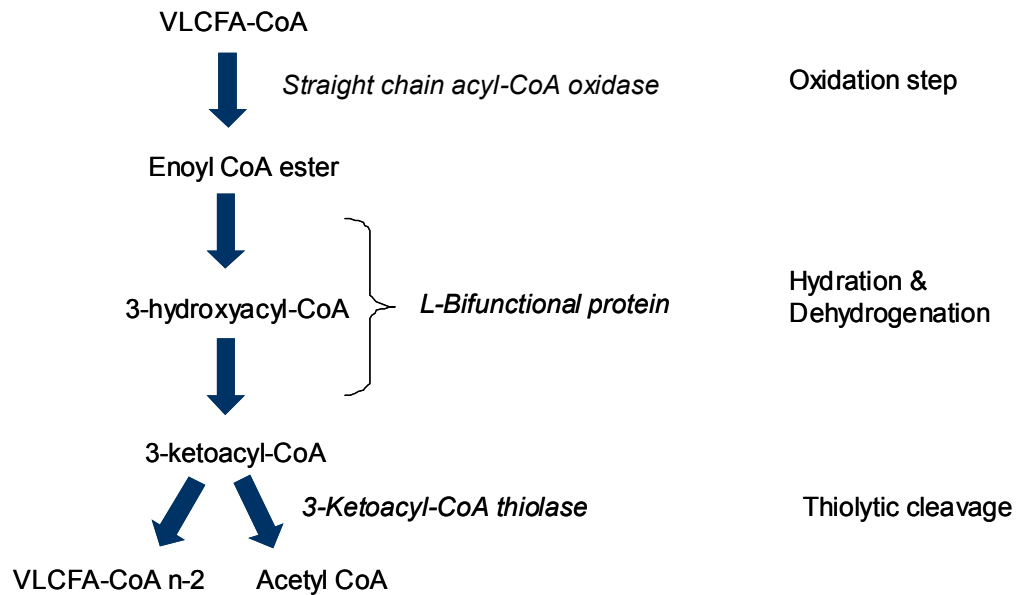


Figure 1-2. Peroxisomal β oxidation of straight chain fatty acid.

There are two complete sets of β oxidation enzymes present in the peroxisome (86). Straight chain acyl CoA oxidase is responsible for the initial oxidation of very long chain fatty acyl CoAs (VLCFA), whereas branched chain acyl CoA oxidase oxidises branched chain fatty acyl CoA. Enoyl CoA esters are then hydrated and subsequently dehydrogenated by the same enzyme. The last step is thiolytic cleavage, performed by sterol carrier protein X in the case of branched chain substrates, whereas straight chain substrates utilise 3-ketoacyl-CoA thiolase.

1.5 CONVERSION OF FATTY ACIDS

The first and rate-limiting enzyme involved in the synthesis of LCPUFAs is $\Delta 6$ desaturase (D6D) (87). D6D introduces a double bond at the $\Delta 6$ position from the

carboxyl end of a fatty acid. This reaction requires molecular oxygen, NAD(P)H and the cytochrome b5 electron transport system (88).

Figure 1-3 shows the series of desaturation and elongations that occur to n-3 and n-6 fatty acids in the endoplasmic reticulum.

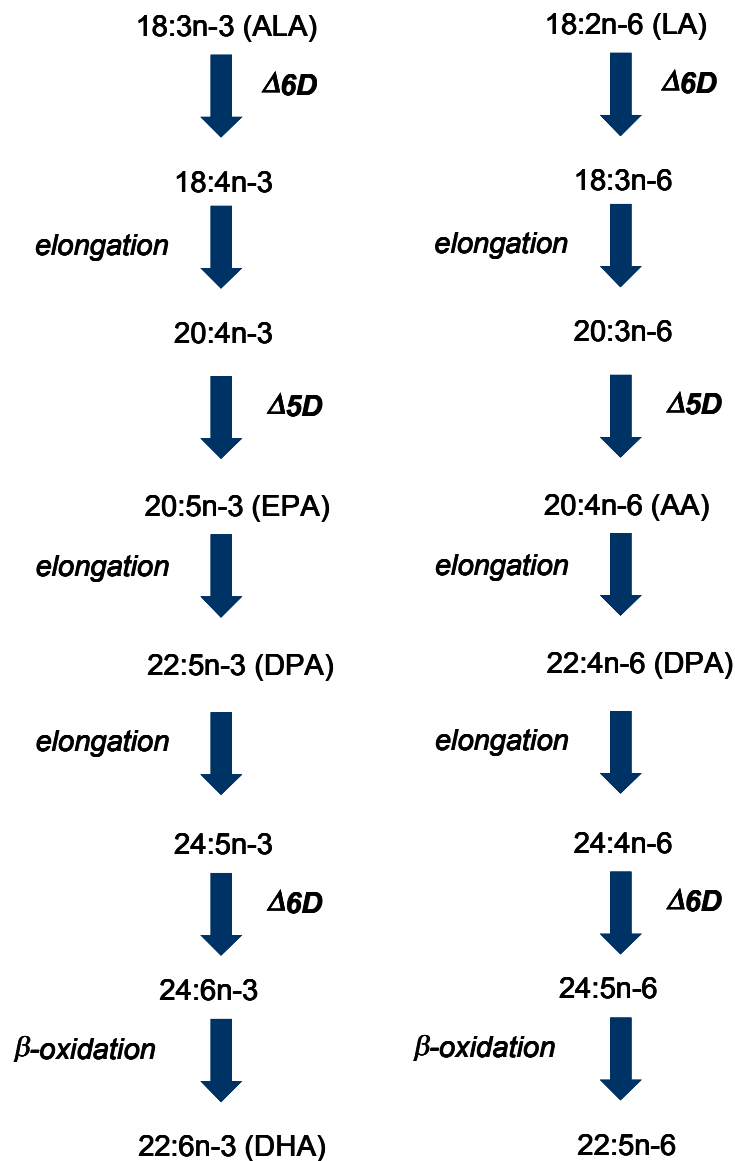


Figure 1-3. Desaturation and elongation of n-3 and n-6 fatty acids.

Elongation and desaturation steps occur in the endoplasmic reticulum. The final β -oxidation step occurs in the peroxisome. $\Delta 6$ desaturase ($\Delta 6D$) is used twice in the synthesis of 22:6n-3 and 22:5n-6 from ALA and LA, respectively.

LA and ALA are substrates for this enzyme and compete for active sites on the enzyme. The products of $\Delta 6$ desaturation, γ -linolenic acid (GLA, 18:3n-6) and stearidonic acid (18:4n-3), are elongated to dihomo- γ -linolenic acid (20:3n-6) and 20:4n-3, respectively. Elongation is a multi-enzyme process that adds two carbon units to the carboxyl end of the fatty acid chain (79;89). Biochemical characterisation of mammalian elongation systems has indicated that the 'elongase' step in the conversion of fatty acids actually consists of four enzymatic reactions, including condensation, β -keto-reduction, dehydration and enoyl reduction. The predominant pathway for fatty acid elongation also occurs in the endoplasmic reticulum. These enzymes work in concert with the desaturases and use malonyl CoA and fatty acyl CoA as substrates for two carbon additions to the fatty acids (79). In the cell, there exist multiple microsomal elongation systems with different chain length specificity (89;90). Six distinct elongases have been described, each with overlapping substrate specificity. The chain elongation system accepts a wide variety of saturated and unsaturated fatty acids as primers for chain elongation (89).

The products of elongation, 20:3n-6 and 20:4n-3, are then further desaturated to the 20 carbon fatty acids by $\Delta 5$ desaturase (D5D) to generate arachidonic acid (AA, 20:4n-6) and EPA. D5D accepts fatty acids with their first double bond at position eight (89) and introduces a double bond at the $\Delta 5$ position along the carbon fatty-acyl chain. AA and EPA are further elongated to 22:4n-6 and 22:5n-3, respectively, and finally to their respective elongation products, 24:4n-6 and 24:5n-3. These final elongation products then become substrates for D6D. Several studies have indicated the utilisation of D6D by LA and ALA, and 24:4n-6 and 24:5n-3. The conversion of LA to 18:3n-6 and 24:5n-3 to 24:6n-3 has been demonstrated in COS-7 cells expressing the wild type rat D6D (91). Human skin fibroblasts isolated from a

patient with clinical evidence of an inherited abnormality in PUFA metabolism, including reduced plasma levels of AA and DHA compared with normal human plasma, showed a reduced conversion of labelled LA to AA, 24:4n-6 and 22:5n-6; and ALA to 24:5n-3 and DHA, compared with normal human fibroblasts (92). Northern blot analysis indicated that the reduced conversion of D6D substrates may be due to the 81-94% decrease in D6D mRNA content compared with normal human fibroblasts (92). de Antueno *et al.* (2001) (93) have also demonstrated that a single human D6D is active on LA, ALA, 24:4n-6 and 24:5n-3. Therefore, it is generally accepted that D6D is used twice in the conversion of ALA to DHA (and LA to 22:5n-6).

Until recently, a $\Delta 4$ desaturase was suspected in the synthesis of DHA and its n-6 homologue, 22:5n-6, from 24:6n-3 and 24:5n-6 respectively. In 1991, Sprecher's group demonstrated that DHA was synthesised by further $\Delta 6$ desaturation of 24:5n-3 to 24:6n-3 followed by peroxisomal β -oxidation to DHA (94). These results were recently confirmed by D'andrea *et al.* (2002) (95). It is now accepted that the final step in the synthesis of DHA (and 22:5n-6) from precursor fatty acids involves partial β -oxidation to remove two carbons in the peroxisome. This process relies on the movement of acyl fatty acid groups from the endoplasmic reticulum to the peroxisome, a process that must be tightly regulated (96).

Studies in patients with disorders of peroxisomal β -oxidation (Zellwegers syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease) indicate the involvement of this organelle in the synthesis of DHA. Patients with Zellwegers syndrome, who lack functional peroxisomes, have reduced levels of DHA in tissues, especially in brain and retina, but also in liver, kidney and blood (97-99). Moore *et*

al. (1995) (100) reported that normal human fibroblasts metabolised 1-¹⁴C ALA to labelled 24:5n-3, 24:6n-3 and DHA. In contrast, fibroblasts from patients with Zellwegers syndrome metabolised labelled ALA to 24:5n-3 and 24:6n-3 but not to DHA. Likewise, 3-¹⁴C 22:5n-3, 3-¹⁴C 24:5n-3 and 3-¹⁴C 24:6n-3 were all metabolised to DHA in normal fibroblast but not in fibroblasts from patients with Zellwegers syndrome. When fibroblasts from patients with Zellwegers syndrome were supplemented with 1-¹⁴C DPA, large amounts of 24:6n-3 accumulated but did not proceed further to form DHA (99). Animal models of Zellwegers syndrome, such as the PEX5 knockout mouse model, also support the role of the peroxisome in the synthesis of DHA (101). These animals have a 40% reduction in the level of DHA in brain tissue compared with the wild type (101). Fatty acid metabolic studies using peroxisomal deficiencies also demonstrate that the synthesis of DHA from ALA involves the formation of 24 carbon polyunsaturated intermediates followed by partial β -oxidation in the peroxisome to DHA, as described above. These studies highlight the importance of the peroxisome and peroxisomal β -oxidation in the synthesis of DHA from ALA. The involvement of two different organelles in the biosynthesis of DHA implies that intracellular movement of fatty acids occurs between the endoplasmic reticulum and the peroxisome, representing a further regulatory site in the synthesis and accumulation of DHA and the level of DHA in cell membranes (102).

Figure 1-4 is a circular representation of the desaturation-elongation pathway, depicting the complexities involved in fatty acid conversion and emphasises the use of D6D twice in the conversion of ALA (and LA) to DHA (and 22:5n-6). This pathway indicates that there are four competing substrates for D6D. Therefore, D6D is used twice in the synthesis of DHA from ALA. It has been reported that the 18

carbon fatty acid substrates are preferred over the 24 carbon fatty acids (87;95). The endogenous synthesis of LCPUFA from precursor fatty acids is therefore dependent on the competition between D6D substrates. The involvement of several enzymatic steps in the conversion of PUFA to LCPUFA implicates the desaturation-elongation pathway as a potent regulator of the fatty acid composition of cell membranes, and particularly the conversion of ALA to DHA.

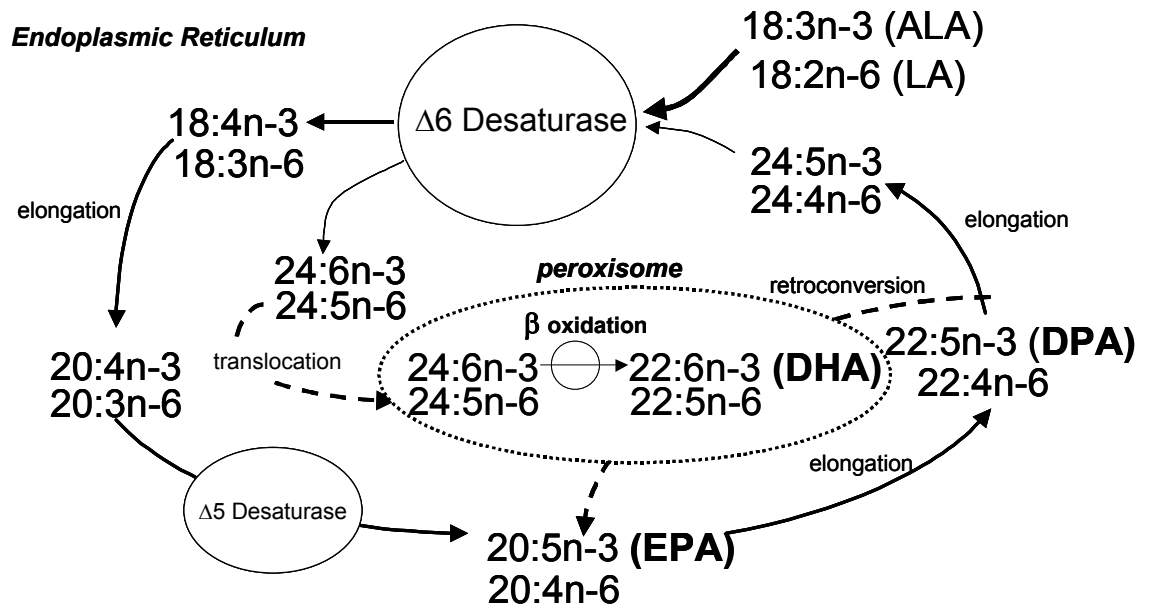


Figure 1-4. Circular representation of n-3 and n-6 fatty acid conversion.

Elongation and desaturation occurs in the endoplasmic reticulum. The final step in the synthesis of docosahexaenoic acid (DHA) and 22:5n-6 from α -linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively, occurs in the peroxisome. The diagram indicates that there are four competing substrates for $\Delta 6$ desaturase and that $\Delta 6$ desaturase is used twice in the synthesis of DHA and 22:5n-6 from ALA and LA, respectively. EPA; eicosapentaenoic acid, DPA; docosapentaenoic acid.

1.5.1 $\Delta 6$ desaturase

D6D was first cloned from the cyanobacterium; *Synechocystis*, and has since been cloned from the wild herb; *Borage officinalis* (103), the nematode; *Caenorhabditis elegans* (104), mice (105), rats (106) and humans (105). D6D is localised on human chromosome 11 (11q12-q13.1) and is expressed in nearly all human tissues, with the greatest activities found in the liver, heart and brain (107). Structurally, the enzyme

has two membrane spanning domains and three Histidine-box motifs that are characteristic of membrane-anchored desaturases and a cytochrome b5 domain (88). Research has shown that D6D enzymatic activity and mRNA expression varies with hormonal and nutritional manipulation (80;107-109).

1.5.1.1 $\Delta 6$ desaturase activity

The activity of the microsomal enzyme, D6D, has been examined using isolated rat liver microsomes (110;111). In the presence of co-factors, such as ATP, coenzyme A and NADH, microsomal preparations have been used to demonstrate the conversion of radiolabelled substrate fatty acids to their immediate products. For example, the rate of conversion of ALA to its immediate product, 18:4n-3, was greater than the conversion of LA to GLA (110). Studies show the greatest activity of D6D when ALA was the substrate, followed by LA and OA (110;111). 24:4n-6 and 24:5n-3 are also substrates for D6D and are desaturated to yield 24:5n-6 and 24:6n-3, respectively, however, kinetic studies indicate a greater capacity for D6D to desaturate 18 carbon fatty acids over the 24 carbon fatty acids (112). One study using isolated microsomes from human fetal liver has shown a similar pattern of activity (113). The enzyme kinetics of D6D will influence the conversion of substrate fatty acids and their availability for incorporation into cellular lipids or β -oxidation.

The fatty acid composition of the background diet also modulates D6D activity. Voss *et al.* (1991) (94) has shown that the desaturation of LA and 24:5n-3 was significantly greater in animals maintained on a fat-free diet compared with those fed rat chow containing PUFA (94). The level of DHA converted from DPA in microsomes isolated from rats fed a fat free diet was seven times that observed in rats

fed a chow diet (94). It has also been shown that D6D activity correlates with the amount of D6D protein. In rats treated with clofibric acid, an increase in the activity of D6D was attributed to the induction of the protein as administration of an inhibitor of protein synthesis (cycloheximide) prevented the increased conversion of LA to GLA (114). A more direct measure of the level of protein is the use of specific antibodies to quantify the protein. Using affinity purified anti-rat D6D rabbit serum, Shoji *et al.* (2003) (115) showed that the increased conversion of [¹⁴C] LA to [¹⁴C] GLA induced by treatment with gemfibrozil correlated to an increase in the level of D6D protein. These studies indicate that the activity of the key enzyme in the conversion of fatty acids can be modulated by the diet and thereby affect the conversion of substrates and influence the fatty acid composition of cell membranes.

1.5.1.2 mRNA expression of $\Delta 6$ desaturase

Dietary studies indicate that hepatic D6D mRNA expression is induced by diets low in LA and ALA and suppressed by diets rich in vegetable or marine oils (80;105;107). D6D mRNA expression may be used as a surrogate marker for the activity of the enzyme as some studies have shown that the suppression of D6D mRNA expression by dietary PUFA is associated with reduced D6D activity in rodents (107;108). Tang *et al.* (2003) (107) demonstrated that both D6D mRNA expression and activity was reduced by 60% in rats fed a high carbohydrate diet supplemented with 10 weight (wt) % safflower oil compared with those fed a fat-free diet or those supplemented with 10 wt % triolein, a triglyceride composed of oleic acid (OA, 18:1n-9). Also, rats fed a 10 wt % fish oil supplemented diet as compared with a chow fed diet resulted in a 40% reduction in D6D activity which correlated with a decrease in hepatic mRNA D6D expression (108).

Nuclear run-on analysis suggests that modulation of D6D mRNA expression by PUFA is due to regulation at the transcriptional level (107). Fatty acids are thought to bind to peroxisome proliferators-activated receptors (PPARs) that bind to peroxisome proliferators-activated receptor response elements (PPRE) on the D6D gene (116-118). The group of lipid lowering drugs, known as fibrates, are also agonists for PPAR and modulate fatty acid metabolism. PPREs have been identified on genes which are components of the peroxisomal long chain fatty acid β -oxidation pathway including the long chain fatty acyl CoA oxidase, the enoyl CoA hydratase and the 3-oxoacyl CoA thiolase (reviewed in (119)). However, there appears to be species differences in the control of fatty acid metabolism and their response to PPAR agonists. In rodents, PPAR agonists cause hepatic peroxisome proliferation, hepatomegaly and, with prolonged administration, hepatocarcinogenesis, in addition to the induction of the fatty acid oxidation enzymes (120). Unlike isolated hepatocytes and tumour derived liver cell lines from rats and mice, human hepatocytes and HepG2 cells do not exhibit peroxisome proliferators-dependent peroxisome proliferation or induction of peroxisome proliferator-associated genes (114). Species differences in the response to synthetic PPAR agonists may also extend to the response to natural PPAR agonists, i.e. fatty acids. Therefore, the expression and regulation of D6D and enzymes involved in peroxisomal β -oxidation in rodents may not translate to that in humans. There is considerable research into the effect of dietary fatty acids on the mRNA expression of D6D in rodents, as described below, yet research into human D6D mRNA expression and its regulation is sparse.

Diets containing corn oil, safflower oil (containing 77% of total fatty acids as LA) or fish oil (rich in EPA and DHA) reduced the expression of D6D mRNA compared

with rats maintained on a diet containing either no fat or triolein (105;107). The reduced D6D mRNA expression following LA supplementation may also arise from its conversion to AA. AA has been shown to reduce the expression of D6D mRNA dose-dependently in the human liver carcinoma cell line, HepG2 (107). Therefore, the reduced level of D6D mRNA expression following dietary LA supplementation may be due to LA, AA or both fatty acids. Similarly, the suppressive effect of fish oil on D6D mRNA expression may be attributed to either levels of EPA or DHA. Examination of D6D mRNA expression following dietary supplementation with EPA and DHA individually showed that both fatty acids decreased hepatic D6D mRNA expression and activity compared with controls (109). The effect of ALA or DPA n-3 on D6D mRNA expression has not been investigated. The regulation of D6D mRNA expression will lead to changes in the amount of the enzyme and thereby affect the rate and amount of fatty acid synthesis.

While D6D appears to be the rate-limiting step in the desaturation-elongation pathway, the overall regulation of the pathway is unclear and may contain several points of metabolic control. The importance of the fatty acid composition of cell membranes in disease states suggests that a correlation between the level of D6D mRNA expression and the accumulation of fatty acids in cell membranes needs to be examined further. A thorough description of the effect of D6D mRNA expression and tissue fatty acid composition has yet to be described. One of the aims of this thesis will be to correlate changes in D6D expression with changes in fatty acid composition of cell phospholipids.

1.5.2 Δ 5 desaturase

D5D has been cloned from several animals including humans (121), rats (122) and *C. elegans* (123). The human D5D gene encodes 444 amino acids and possesses 61% amino acid identity and 75% similarity to the human D6D (88). D5D, like D6D, is a microsomal enzyme that is expressed in nearly all human tissues, with the greatest activity found in the liver, heart and brain (121). The D6D and D5D proteins possess several other common features, including an identical polypeptide size, two membrane-spanning domains, and three histidine-rich regions. Examination of the human genome database revealed that the D5D and D6D genes are positioned in reverse sequence orientation to each other on chromosome 11 at 11q12.2 – 11q13 (121). The distance between the exon containing the translation initiation codon for D6D is approximately 11, 000 base pairs (bp) from the exon that contains the translation initiation codon for D5D. Although the specific promoters for the two desaturases have not yet been located, the proximity of the genes suggests that transcription of D5D and D6D may be co-ordinately governed by regulatory sequences within the 11,000 bp region that is common to both genes (121).

1.6 ACYLATION OF FATTY ACIDS INTO CELLULAR LIPIDS

Activated fatty acids may also be substrates for acylation into phospholipids. The cell membrane is composed of a lipid bilayer with phospholipids constituting the most abundant membrane lipid. Fatty acids are important constituents of phospholipids and confer the physical properties of cell membranes. Of the phospholipid classes, phosphatidyl choline (PC) is the most abundant in eukaryotic cell membranes, representing approximately 50% of phospholipid mass (124;125). Phosphatidyl choline (PC) is synthesised by the CDP-choline (Kennedy) pathway (126). The fatty acyl composition of the diacylglycerol molecule utilised by

cholinephosphotransferase in the final step of the CDP-choline pathway determines the fatty acyl array for PC synthesised *de novo* (127). The fatty acid composition of each unique organ or cell suggests that the incorporation of fatty acids into phospholipid classes is highly selective and the conversion of fatty acid precursors to LCPUFA may be tissue-dependent (128-131). However, it must be noted that the level of n-3 LCPUFA in each tissue generally relates to the intake of n-3 LCPUFA although the absolute level is tissue specific.

The selectivity of fatty acids for acylation into membrane lipids represents another potential intracellular control in determining the fatty acid composition of cell membranes. 1-acyl-*sn*-glycero-3-phosphocholine (1-acyl-GCP) is an acceptor of fatty acids that can be used to model esterification of LCPUFA into membrane lipids. In so-called 'mixing studies', 1-acyl-GCP is added to microsomes and peroxisomes isolated from the liver of rats. When labelled EPA, DPA (22:5n-3) or DHA were incubated in this mixture, 53, 5 and 30 η mol, respectively, were esterified into 1-acyl-GPC (132). Neither 24 carbon fatty acid (24:5n-3 or 24:6n-3) were readily esterified into 1-acyl-GCP in these microsomal-peroxisomal mixing studies, indicating that 24:5n-3 and 24:6n-3 are poor substrates for esterification. In cultured hepatocytes, only trace amounts of labelled 24:5n-3 and 24:6n-3 were incorporated into hepatocyte cell phospholipids (94). When these fatty acids are generated in the endoplasmic reticulum, they preferentially move to the peroxisome for further metabolism. Moreover, the rate of acylation of DHA into 1-acyl-GCP was 8.3-fold greater than for DPA (22:5n-3).

Ferdinandusse *et al.* (2003) (133) demonstrated the different rates of acylation between fatty acid substrates in cultured skin fibroblasts. They report that 24:6n-3

was β -oxidised 2.7 times faster than 22:6n-3, while 22:6n-3 was incorporated seven times faster into different lipid classes than 24:6n-3 (133) indicating that the 24 carbon fatty acids preferentially move into the peroxisome for β -oxidation rather than acylation into cellular lipids. The rate of acylation appears to be regulated by the presence of dietary PUFA. The amount of DHA esterified in hepatocyte phospholipids, derived from radiolabelled substrates was about 80-fold greater in animals fed a fat-free diet than for hepatocytes from chow-fed animals containing PUFA (94). There appears to be a preferred metabolic fate for each fatty acid and an inverse relationship between rates of peroxisomal β -oxidation and rates of acylation. These events will particularly influence the synthesis of DHA from fatty acids precursors.

1.7 CONVERSION OF FATTY ACIDS IN ANIMALS

1.7.1 Competition between n-3 and n-6 fatty acids for $\Delta 6$ desaturase

Competition between n-3 and n-6 PUFA for enzymes in the fatty acid synthetic pathway has been demonstrated in animals (134). Mohrhaeur and Holman (1963) (134) fed rats a fat-free diet with daily supplements of either highly purified ethyl linoleate or ethyl linolenate, derivatives of LA (n-6) and ALA (n-3), or both. When dietary LA was held constant, increasing the level of dietary ALA suppressed the tissue content of n-6 products. Conversely, when ALA was held constant, increasing levels of dietary LA suppressed n-3 products (134) indicating that competition between n-3 and n-6 fatty acids exists and that this competition influenced the accumulation of LCPUFA.

1.7.2 Total PUFA affects the $\Delta 6$ desaturation of substrates

The competition between n-3 and n-6 substrates as well as the amount of each substrate appears to be important determinants of substrate desaturation and elongation in the fatty acid synthetic pathway. Lands *et al.* (1990) (135) fed rats diets with different ratios of n-3 and n-6 fatty acids and/or different total amounts of PUFA (ALA and LA). The highest level of EPA in plasma phospholipids was directly related to the energy percent intake of ALA, regardless of the presence of competing n-6 substrates. Interestingly, the highest level of DHA was observed in the liver phospholipids of rats fed a diet with 0.73 en % ALA (and 0.55 en % LA) compared with rats maintained on a diets containing up to 11.26 en % ALA. Despite the significantly lower percent energy intake of ALA, the amount of DHA synthesised and incorporated into liver phospholipids was 12% of total fatty acids compared to 8% in those fed the highest energy percent ALA (11.26 en %). This was mirrored in plasma phospholipids where the level of DHA in rats fed 0.73 en % ALA was 7.48 % total fatty acids compared to 5.68 % total fatty acids in those fed 11.26 en %. Although the amount of ALA available for conversion to DHA was highest in the group receiving 11.26 en % ALA, its conversion and accumulation to DHA appeared to be inhibited.

Other dietary animal interventions demonstrate the paucity in the accumulation of DHA following supplementation with relatively high en % ALA (summarised in

Figure 1-5). Increasing the en % of ALA (constant en % LA) in the diet of hamsters reduced the level of DHA in plasma phospholipids in those fed the high ALA diet (136). However, the diet did contain trace amounts of DHA (0.2% total fatty acids) which may have altered the conversion of fatty acids. There was a proportional

enrichment in EPA and DPA, but not in DHA, which decreased in the group fed the highest energy percent ALA. In hamsters fed the lowest en % ALA (0.37 en %) the level of DHA in plasma phospholipids was 9.88 ± 0.69 % DHA total fatty acids compared to 7.69 ± 0.19 % total fatty acids in the group receiving the highest energy percent of ALA, (14.58 en %) (136). Studies in marmosets also show that low dietary PUFA (2.15 en %) elevated the level of DHA in erythrocytes (137) and cardiac membranes (138) compared with those on a high PUFA diet (9.1 en %). In newborn piglets fed increasing amounts of ALA while keeping the level of LA constant, the accumulation of ALA, EPA and DPA was directly related to the en% of ALA in the formula (139). The accumulation of DHA was highest in piglets fed a formula containing 3.6 en% ALA. In these animals, the level of DHA in plasma phospholipids was 6.36 ± 1.00 % total fatty acids compared to 4.33 ± 0.95 % total fatty acids in animals fed 16 en% ALA (139). The dose-dependent accumulation of EPA suggests that the reduced accumulation of DHA with increasing dietary ALA was not attributable to competition between LA and ALA at the first desaturation step but rather to events beyond the $\Delta 5$ desaturation of 20:4n-3 to EPA (20:5n-3). In those studies where the en % of ALA was increased; there was a counter-intuitive decrease in the level of DHA, suggesting that the enzymes utilised by ALA to synthesise DHA were saturated or inhibited. Hence, high dietary ALA reduces its conversion to DHA. Figure 1-5 shows that low dietary en % ALA results in a greater accumulation of DHA compared with higher en % ALA. The highest accumulation of tissue DHA occurs in a narrow range of en % ALA. In contrast, dietary LA does not appear to have a large influence on the accumulation of DHA in tissues (

Figure 1-5). The total en % of LA and ALA and their ratio to each other appear to be important factors in determining the optimal synthesis of DHA.

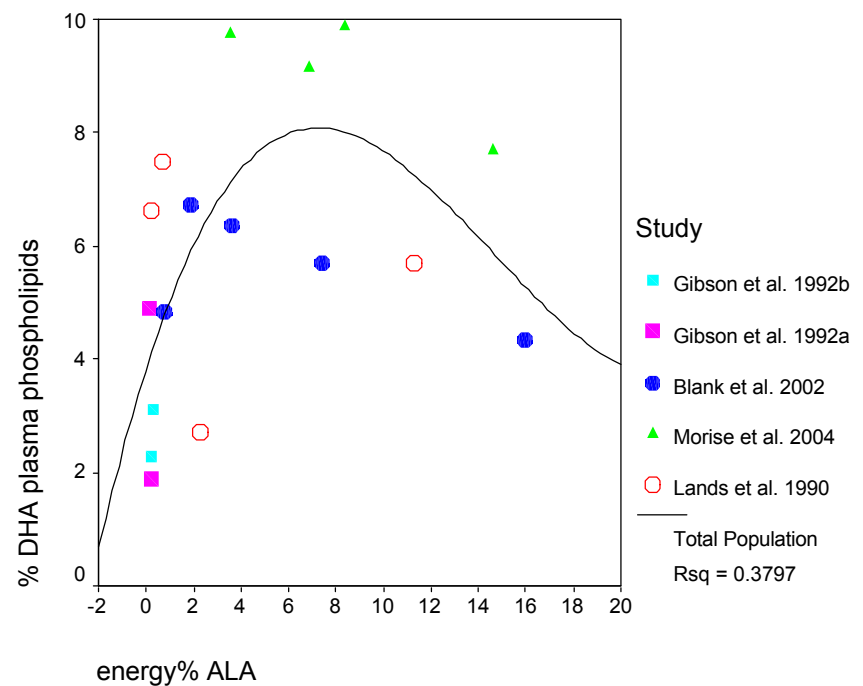
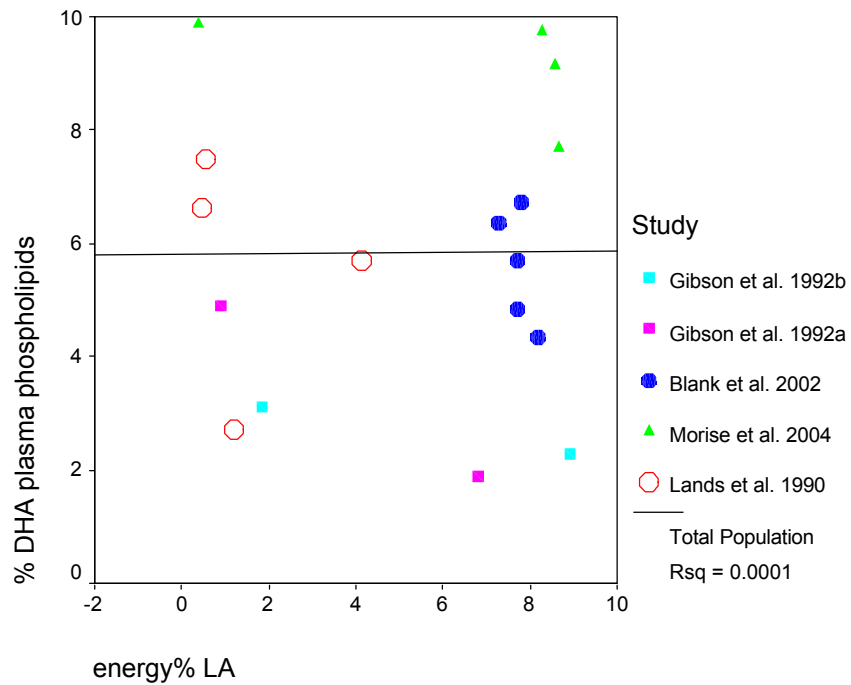


Figure 1-5. Accumulation of docosahexaenoic acid (DHA) in plasma phospholipids of animals fed different energy % of dietary PUFA (linoleic acid (LA) and α -linolenic acid (ALA)). Dietary interventions were performed in rats (Gibson *et al.* 1992a (140), Lands *et al.* 1990 (135)), marmosets (Gibson *et al.* 1992b (137)), pigs (Blank *et al.* 2002 (139)) and hamsters (Morise *et al.* 2004 (136)).

These animal studies suggest that competition exists between fatty acids of the same class for enzymes in the pathway, which may affect the fatty acid composition of cell membranes and the accumulation of LCPUFA from precursors. In the synthesis of DHA from ALA, both ALA and its downstream product, 24:5n-3, utilise D6D. In isolated liver microsomes from rats fed a fat-free diet, increasing concentrations of [1-¹⁴C] ALA inhibited the desaturation of [1-¹⁴C] 24:5n-3. Conversely, increasing amounts of [3-¹⁴C] 24:5n-3 only slightly reduced the amount of ALA that was desaturated, suggesting that ALA is the preferred substrate for D6D (112). The competition between n-3 fatty acids, particularly ALA and 24:5n-3, for enzymes in the pathway also explains the poor accumulation of DHA observed in human intervention studies, described below (see Section 1.8). It was hypothesised that high dietary intakes of ALA depress the synthesis of DHA by inhibiting the desaturation of 24:5n-3 to 24:6n-3 through its competition for D6D.

1.8 CONVERSION OF FATTY ACIDS IN HUMANS

The observation that elevated dietary intakes of ALA in humans results in an increased level of EPA but little or no change in the level of DHA in tissues or plasma (6;30-32;141) (summarised in Table 1-1) (see also (142) for a more extensive review) suggests that the endogenous synthesis and accumulation of LCPUFA from precursor fatty acids is complex. Such reports have led to the general consensus that the synthesis of DHA from ALA is limited in humans. Several studies have reported the effects of feeding high-ALA oils, typically linseed oil, containing approximately 50% ALA, on the fatty acid composition of plasma lipids. Sanders *et al.* (1983) (6) supplemented the diet of healthy human volunteers with 20 ml of linseed oil daily. This is equivalent to approximately 10 g/d ALA (~ 5 en %) and represents four and a half times the recommended dietary intake (as recommended by US National

Institute of Health (8) and Australia (NHMRC 1992, Australia, National Heart Foundation 1999, Australia)). Despite the high intake of linseed oil, only a small proportion of ALA was incorporated into platelet phosphoglycerides and suggests that ALA does not readily accumulate into cell lipids (6). EPA was significantly elevated yet there was no change in the level of DHA, suggesting a lack of conversion further down the desaturation-elongation pathway. The poor conversion of ALA to DHA in this study may result from the competition between ALA and LA for active sites on D6D as LA was not excluded in this study, providing an estimated 4 en %. Indeed, Emken *et al.* (1994) (143) have shown a 40-54% reduction in the conversion of ALA when the dietary intake of LA was doubled.

Another human intervention trial also indicated a limited conversion of ALA to DHA in humans (141). The fatty acid profile of individuals on a typical Western diet, rich in n-6 fats was compared with those maintaining a high n-3 (ALA) diet by using linseed oil and linseed spread and avoiding n-6 rich foods for four weeks. The amount of ALA consumed correlated with the level of ALA and EPA in plasma, neutrophils, mononuclear cells, erythrocytes and platelet phospholipids but inversely correlated with the level of DHA. However, participants were allowed two fish meals/week providing a source of EPA and DHA which may have affected the level of DHA in tissues due to the mechanisms mentioned previously.

De Groot *et al.* (2004) (144) supplemented pregnant women with 2.8 g/d of ALA contained in a margarine spread and measured the level of n-3 LCPUFA following supplementation. Plasma concentrations of EPA and DPA increased by 30% and 15% respectively but DHA did not differ between the ALA supplemented and control groups. In a linseed oil supplementation trial in vegetarian men,

consumption of 15.4 g/d of ALA resulted in 7-, 4.5-, and 1.5-fold increases of plasma ALA, EPA and DPA, respectively, with no change in DHA (145). The studies presented in Table 1-1 indicate that there is a limitation in the accumulation of DHA from ALA in humans. The increased accumulation of DPA in tissues following supplementation with ALA suggests that the limitation may be at a point beyond the conversion of ALA to DPA, for instance, at the second $\Delta 6$ desaturation step in the synthesis of DHA from ALA, the translocation of substrates (24:6n-3) from the endoplasmic reticulum to the peroxisome, peroxisomal β -oxidation or translocation back to the endoplasmic reticulum for membrane lipid biosynthesis.

1.8.1 Conversion of fatty acids in humans: stable isotope studies

The interpretation of human intervention studies is problematic due to the wide availability of other fatty acids in the diet and the potential for dietary fatty acids to modulate the conversion of other fatty acids to LCPUFA (146-148). By administering a short term administration of a bolus of ALA labelled with a stable isotope and monitoring the conversion of ALA to LCPUFA this problem can be briefly circumvented. Table 1-1 summarises several studies using stable isotopes to estimate the conversion of ALA to EPA and DHA in humans. Emken *et al.* (1994) (143) described the total percent conversion of deuterated ALA to deuterated EPA, DPA and DHA in a group of healthy males at 6.0%, 3.5% and 3.8%, respectively (149). As human fatty acid supplementation trials are affected by the influence of the background diet, so too are stable isotope studies (146;148). The study by Pawlosky *et al.* (2003) (147) demonstrates the effect of background diet on the conversion of labelled ALA, with reduced conversion of DPA to DHA being reported following consumption of a fish-based diet compared with a beef-based diet (147). The influence of gender on the conversion of fatty acids was also evident

when the fractional conversion of DPA to DHA was calculated separately for men and women. The reduced conversion of DPA to DHA was only observed in women consuming the fish-based diet (147). Differences in study design, background diet and the description of results contribute to the heterogeneity of the studies presented in Table 1-1. However, stable isotope studies indicate that in free-living populations the conversion of ALA to DHA is marginal.

1.8.2 Conversion of fatty acids: differences between women and men

Differences in the percent conversion of ALA to n-3 LCPUFA have been described between women and men and suggest that hormonal control may be involved in regulating the expression and activity of desaturation and elongation enzymes. The total percent conversion of deuterated ALA to deuterated EPA, DPA and DHA was reportedly 6.0%, 3.5% and 3.8%, respectively, in a group of healthy males (143). Another study reported that the conversion of [¹³C] labelled ALA to DHA was undetectable in men (31), while the conversion in women was 9% (32). A study in volunteers consuming a strictly controlled diet, containing no n-3 fatty acids from fish and a constant amount of ALA, for three weeks found that the amount of DHA derived from ALA was higher in women (14.6 ± 3.9%) than in men (150). In male-to-female gender reassignment patients the level of DHA in plasma cholesterol esters increased after administration of ethinyl estradiol plus cyproterone acetate (drugs which mimic female hormones) for four months. In female-to-male gender reassignment patients, the level of DHA was reduced after four months of testosterone administration (150). The increased accumulation of DHA in women appears to be due to the influence of oestrogen on fatty acid synthesis. The increased conversion of [¹³C] ALA to [¹³C] DHA in women compared to men suggests up-

regulation of the desaturation and elongation pathway and also increased partial β -oxidation in the peroxisome, perhaps by oestrogen. This is consistent with the observation that women taking 30-35 μg ethinyloestradiol/d in a contraceptive pill, which represents an increase in oestrogen exposure compared with the menstrual cycle, had 2.5-fold greater conversion of [^{13}C] ALA to DHA than those who did not take synthetic oestrogens (31).

Ingested ALA has several metabolic fates. Apart from being a substrate for fatty acid synthesis, a major catabolic route of metabolism of ALA is complete oxidation to CO_2 . In a study in humans, between 16 and 20% of ALA was expired as CO_2 in 12 h (151). In addition to the excretion of CO_2 on breath, carbon released from fatty acid β -oxidation may be recycled and used for fatty acid synthesis *de novo*. Citrate released from mitochondria into the cytosol is cleaved to form acetyl-CoA and oxaloacetate. The acetyl CoA is then available for fatty acid biosynthesis. There appears to be reciprocal relationship between partitioning of [^{13}C] ALA towards β -oxidation and carbon recycling into saturated and monounsaturated fatty acids, and conversion to EPA, DPA and DHA. Women preferentially partition fatty acids towards synthesis whereas men use fatty acids as an energy source to a greater extent than women (152). It is well accepted that the presence of other fatty acids in the diet affects the conversion and accumulation of LCPUFA from precursor fatty acids. Studies which have examined the conversion of labelled fatty acids to their long chain metabolites in women and men indicate that the conversion of fatty acids is also subject to hormonal regulation.

Reference	Study population	Dietary intervention	Duration (weeks)	Outcome
James <i>et al.</i> 2003 (153)	45 M + post-menopausal F	0.75 g for 3 weeks then 1.5 g for the following 3 weeks of ALA, SDA or EPA ethyl ester	6	<ul style="list-style-type: none"> Increased concentration of EPA and DPA in plasma and erythrocyte phospholipids following supplementation with SDA and EPA. No change in the concentration of DHA in plasma phospholipids or erythrocytes with any dietary group.
Mantzioris <i>et al.</i> 1995 (141)	30 M	Control (Typical Western diet, high in LA) versus High-ALA group (13.0 g/d ALA)	4	<ul style="list-style-type: none"> Intake of ALA correlated with the concentration of EPA in plasma, neutrophils, mononuclear cells, erythrocytes and platelet phospholipids but inversely correlated with DHA.
Walace <i>et al.</i> 2003 (154)	40 M + F	Placebo (palm oil-soyabean oil (80:20 w/w), 3.5 g/d ALA, 0.44 g/d EPA+DHA, 0.94 g/d EPA+DHA, or 1.9 g/d EPA+DHA)	12	<ul style="list-style-type: none"> Increased intake of ALA did not increase the concentration of ALA in plasma phospholipids but increased the concentration of EPA by 60%. No change in the concentration of DHA in plasma phospholipids following supplementation with ALA The concentration of EPA and DHA in plasma phospholipids was increase in all subjects consuming EPA+DHA.
Harper <i>et al.</i> 2006 (155)	49 F + 7 M (chronically ill)	Control (5.2 g/d olive oil) versus 5.2 g/d flaxseed oil (3.0 g/d ALA)	26	<ul style="list-style-type: none"> 70 % increase in the concentration of ALA in plasma lipids in the group receiving flaxseed oil, concomitant with a 60% increase in EPA and a 34% increase in DPA. The level of DHA in plasma lipids was unaffected in both groups
De Groot <i>et al.</i> (144)	58 F (pregnant)	From week 14 of gestation to delivery, pregnant women consumed 2.8 g/d ALA and 9.0 g/d LA or 10.9 g/d LA (control).	26	<ul style="list-style-type: none"> Plasma concentrations of EPA and DPA increased by 30% and 15% respectively but DHA did not differ between the ALA supplemented and control groups.

Reference	Study population	Dietary intervention	Duration (weeks)	Outcome
Li <i>et al.</i> 1999 (145)	17 M (vegetarians)	Moderate-ALA diet (LA:ALA 3:1, 13.1 g/d LA, 3.7 g/d ALA) versus high-ALA diet (LA:ALA 1:1, 17.4 g/d LA, 15.4 g/d ALA).	4	<ul style="list-style-type: none"> • Significant increase in the level of EPA and DPA in platelet phospholipids in subjects consuming the high-ALA diet. • Significant increase in ALA and EPA in plasma phospholipids in subjects consuming the high-ALA diet. • The level of DHA in platelets and plasma phospholipids was unaffected by both diets.
Stable isotope studies				
Vermunt <i>et al.</i> 1999 (146)	15 M + F	OA-rich diet, ALA-rich diet (6.8 g/d), EPA+DHA-rich diet (1 g EPA + 0.6 g DHA). Following 6 weeks of the dietary intervention, 45 mg [¹³ C] ALA methyl ester was administered.	6	<ul style="list-style-type: none"> • [¹³C] enrichment of ALA, EPA, DPA and DHA was reduced in ALA-rich and EPA+DHA-rich diets compared with OA-rich diet. • Minimal [¹³C] enrichment of EPA, DPA and DHA in the EPA+DHA-rich diet.
Goyens <i>et al.</i> (156)	15 M + 15 F	Control (LA:ALA 19:1; 7 en% LA, 0.4 en% ALA.), Low LA (LA:ALA 7:1; 3 en% LA, 0.4 en% ALA), High ALA (LA:ALA 7:1; 7 en% LA, 1.1 en% ALA). At 19 and 61 d, subjects ingested 11.4 g [¹³ C] ALA.	61 d	<ul style="list-style-type: none"> • The level of ALA and EPA in plasma phospholipids in subjects consuming the low-LA and high-ALA diets increased significantly compared to the control group. • The level of DPA in plasma phospholipids increased significantly in subjects consuming the high-ALA diet. • The level of DHA in plasma phospholipids was not significantly different between dietary groups. • 5.2% ALA was converted to DPA in subjects consuming the low-LA diet.

Reference	Study population	Dietary intervention	Duration (weeks)	Outcome
Stable isotope studies continued				
Pawlosky <i>et al.</i> 2003 (147)	5 M + 5 F	Beef-based diet (no seafood or seafood products) for 3 weeks followed by a fished-based diet for another 3 weeks, after which 1 g [² H] ALA ethyl ester was administered.	6	<ul style="list-style-type: none"> Elevated concentration of EPA and DHA in plasma lipids during the fish-based diet compared with beef-based diet. Reduced conversion of DPA to DHA following consumption of fish-based diet compared with the beef-based diet.
Pawlosky <i>et al.</i> (157)	4 M + 4 F	Beef-based diet for 21 d. During the final week of the dietary intervention, 1 g [² H] ALA ethyl ester was administered.	3	<ul style="list-style-type: none"> 0.2% [²H] ALA converted to EPA, 0.13% [²H] ALA converted to DPA, 0.05% [²H] ALA converted to DHA.
Emken <i>et al.</i> 1999 (148)	6 M	High-DHA diet (6.5 g/d DHA) versus low-DHA diet (<0.1 g/d DHA) for 90 d prior to administration of [² H] ALA triglyceride.	90 d	<ul style="list-style-type: none"> DHA supplementation reduced the conversion of ALA to EPA and DHA by 76 and 88% respectively.

Reference	Study population	Dietary intervention	Duration (weeks)	Outcome
Stable isotope studies continued				
Emken <i>et al.</i> 1994 (143)	7 M	SAT diet containing 15.1 g LA versus PUFA diet containing 29.8 g LA for 12 d prior to administration of a mixture of [² H] ALA and LA triglycerides.	12 d	<ul style="list-style-type: none"> The total percent conversion of deuterated ALA to deuterated EPA, DPA and DHA was 6.0%, 3.5% and 3.8%, respectively, in all subjects. Conversion of [²H] ALA and LA was reduced by 40-54% when dietary intake of LA was increased from 15.1 g/d to 29.8 g/d.
Burdge <i>et al.</i> 2002 (31)	6 F	0.7 g [¹³ C] ALA free fatty acid	Once only administration	<ul style="list-style-type: none"> Conversion of ALA to EPA, DPA and DHA was 21, 6 and 9 %, respectively, in plasma lipids.
Burdge <i>et al.</i> 2002 (32)	6 M	0.7 g [¹³ C] ALA free fatty acid	Once only administration	<ul style="list-style-type: none"> Conversion of ALA to EPA and DPA was 8 and 8%, respectively, in plasma lipids. Conversion of ALA to DHA was not detected.

Table 1-1. Accumulation of n-3 LCPUFA in human populations: a summary of current evidence.

1.9 CONVERSION OF FATTY ACIDS *IN VITRO*

One of the major limitations of animal and human studies is the influence of other dietary fatty acids on the synthesis of DHA from ALA and the difficulty in controlling their concentration. *In vitro* models of fatty acid metabolism allow the conversion of individual fatty acids to be examined in isolation of other fatty acids. Several epithelial cell lines have been used to demonstrate the capacity of cell lines to mimic *in vivo* fatty acid metabolism. De Antueno *et al.* (2001) (158) reported the conversion of radiolabelled LA to 18:3n-6 in the ZR-75-1 (human mammary carcinoma) cell line. Following $\Delta 6$ desaturation, GLA was elongated to 20:3n-6 and further desaturated ($\Delta 5$) to 20:4n-6 and then elongated to 22:4n-6 (158), following the desaturation-elongation pathway described in Figure 1-3. Northern blot analysis of the ZR-75-1 cell line indicated the presence of both $\Delta 5$ and $\Delta 6$ desaturase transcripts (158) and indicates the usefulness of this cell line as an *in vitro* model of fatty acid conversion.

The competition between LA and ALA for common enzymes in the desaturation-elongation pathway reported *in vivo* is also demonstrated *in vitro*. When unlabeled fatty acids, ALA, EPA, DHA, LA and AA were added to cell culture media of intestinal CaCo-2 cells in the presence of radiolabelled ALA and LA, the metabolism of labelled PUFA to LCPUFA was reduced (159). The addition of unlabelled AA and EPA reduced the conversion of radiolabelled ALA to EPA by 75.8% and 90.3%, respectively. As the ratio of LA to ALA increased, conversion and incorporation of labelled ALA into CaCo-2 cell lipids decreased (159). Further elongation and desaturation of radiolabelled DPA to radiolabelled DHA was not detected (159). However, Dokko *et al.* (1998) (160) examined the metabolism of labelled LA in

HepG2 cells but did not observe any conversion to AA. This was also reported by Marra *et al.* (1992) (161) in a study using the SK-Hep1 cell line. The inability of these cell lines to convert LA to AA may have resulted from the development of essential fatty acid deficiency, which has been suggested to cause reduced conversion of n-6 fatty acids to their long chain derivatives (162). Melin and Nilsson (1997) (162) reported that an essential fatty acid deficient state occurs after five to ten days of culture in the absence of essential fatty acids. In both reports, cells were confluent and were cultured for more than five days prior to fatty acid supplementation, which may explain the inability to convert LA to AA. The conversion of LA to AA has been described in other cell lines cultured over a shorter time period (158). In the SK-Hep1 cell line, [¹⁴C] ALA was converted to 20:5n-3 and 22:5n-3 but its conversion to DHA was not detected (161). The conversion of fatty acids and the competition between fatty acid substrates reported in cell lines aligns with that described *in vivo* and suggests that *in vitro* cell systems may provide a tool in examining the potential limitations in the conversion of ALA to DHA.

1.9.1 mRNA expression of Δ 6 desaturase *in vitro*

Several *in vitro* studies have utilised transformed liver cell lines to demonstrate the conversion of labelled ALA to EPA, DPA and DHA (163) and to examine the mRNA expression of the key enzymes involved in this conversion, and more specifically, D6D. Nara *et al.* (2002) (164) showed a ~50% decrease in D6D mRNA expression in HepG2 cells in the presence of AA, EPA and DHA, compared to cells without PUFA supplements or cells supplemented with oleic acid (18:1n-9), in agreement with that reported in animals (80;105;107). Tang *et al.* (2003) (107) showed a dose-dependent decrease in D6D mRNA expression in HepG2 cells supplemented with AA. These studies indicate that the expression of D6D is

modulated by fatty acids. However, there is a lack of studies that thoroughly investigate the effect of D6D mRNA expression on the composition of cell membranes and whether this is in fact the limiting step in the conversion of ALA to DHA.

HepG2 cells are a useful cell system to examine the influence of D6D mRNA expression and fatty acid supplementation on the fatty acid composition of cell membranes. The human hepatoma cell line, HepG2 is of particular interest as the liver is the primary site for the synthesis of fatty acids. The HepG2 cell line exhibits many differentiated functions of human liver cells, including expression of secretory proteins such as albumin, transferrin and fibrinogen (165;166) and retains many normal hepatic metabolic functions, including lipoprotein and apolipoprotein synthesis and cholesterol metabolism (160;167). The use of this cell line, however, is not without its limitations. Like many cell lines, HepG2 cells are derived from neoplastic tissues and as such are de-differentiated in some way from the original hepatocyte (168). Primary human hepatocytes may provide an alternative but are associated with limited supply, heterogeneity and quality (168;169), including the loss of liver-specific functions with time in culture (165). The use of the HepG2 cell line in previous studies however does suggest its use as a suitable model of fatty acid conversion and will allow direct comparison between the data produced in this thesis with other studies in the field.

1.10 AIMS

Numerous intervention studies have attempted to elevate the level of DHA in tissues by increasing dietary ALA without success. While it is generally accepted that the conversion of ALA to DHA is limited, there is little understanding as to the mechanisms governing this limitation. There are several plausible explanations for

the disparity between the intake of ALA and its conversion to DHA *in vivo*. Regulation of the concentration of DHA in tissues and plasma may involve simple substrate competition between n-3 and n-6 PUFA for common enzymes in the pathway, preferential esterification of certain fatty acids into cell lipids or peroxisomal β -oxidation or mitochondrial oxidation of fatty acids or a combination of these processes. The expression of the D6D mRNA may also influence membrane fatty acid composition by affecting the amount of D6D protein available for the conversion of fatty acids.

Much of our understanding of fatty acid conversion and accumulation into cell lipids has been gained from *in vivo* and *in vitro* studies. However, interpretation of *in vivo* studies is often limited by the multitude of other dietary fatty acids which can influence the conversion of fatty acids (147). Animal studies which examine the conversion and accumulation of a single, pure fatty acid do so by depleting the diet of all fat, thereby inducing essential fatty acid deficiency and altering the metabolic state of the animal (107;134). The altered metabolic state will affect the metabolism of fatty acids and the fatty acid composition of cell membranes. *In vitro* studies examining the conversion of labelled fatty acid substrates have demonstrated the kinetics of D6D yet are limited in their ability to show gross changes in the fatty acid composition of cell membranes as minute quantities of labelled fatty acids are administered and measured and radioactivity may not reliably be converted to mass. Moreover, it has been shown that the phospholipid distribution of incorporated labelled fatty acids may differ markedly from that of endogenous fatty acids (170). Furthermore, supplementation of animals with individual, pure fatty acids would be prohibitively expensive and more so in humans.

In vitro studies are advantageous in allowing cells to be supplemented with fatty acids individually and exclusively of other fatty acids. However, there is limited *in vitro* data which thoroughly describes the fatty acid composition of cell membranes following supplementation with individual fatty acids. Of the limited data available, there is some discrepancy between the conversion and accumulation of fatty acids *in vitro* with that compared with *in vivo* and requires further investigation.

This thesis will use the HepG2 cell line to model the accumulation of fatty acids into cell membranes *in vivo*. This cell line reportedly retains many of the hepatic metabolic functions of the normal human liver (160;165-167), the primary site for the conversion of ALA to EPA and DHA. Changes in the fatty acid composition of HepG2 cell phospholipids following supplementation with single fatty acids will be examined in an attempt to gain insights into the potential regulatory steps in the conversion of fatty acids *in vivo*. The accumulation of fatty acids (a net effect of conversion and incorporation) into cell phospholipids following supplementation with individual fatty acids is presented in this thesis. Measuring the fatty acid composition of HepG2 cell phospholipids following fatty acid supplementation demonstrates mass changes in the fatty acid composition of cell phospholipids rather than the conversion of a fatty acid substrate to their immediate product, as in stable isotope studies. In this way, an *in vitro* system may be used to model the mass changes in the fatty acid composition of tissues *in vivo* that may occur following supplementation with fatty acids. This thesis aims to:

- (i) examine the conversion of PUFA to LCPUFA through the accumulation of LCPUFA in HepG2 cell phospholipids following incubation with PUFA; and
- (ii) delineate the possible regulatory steps in the conversion of ALA, and other n-3 fatty acids, to DHA.

2 Materials and Methods

This chapter describes in detail the most utilised methods of this thesis, namely cell culture of HepG2 cells and fatty acid analysis. Modifications to these methods are described in each chapter as appropriate.

2.1 MATERIALS

Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin were purchased from SAFC Biosciences, Victoria, Australia. Free fatty acids (FFA) and authentic lipid standards were obtained from Nu-Check-Prep Inc (Elysian, MN, USA). The C17:0 phospholipid (diheptadecanoyl) and C17:0 triglyceride (triheptadecanoin) internal standard was from Sigma (St. Louis, MO, USA) and the C17:0 cholesterol ester (cholesteryl heptadecanoate) internal standard were from Nu-Check-Prep Inc. (Elysian, MN, USA). BSA, dimethyl sulfoxide (DMSO) and trypan blue solution were from Sigma (St. Louis, MO, USA). Tissue culture ware was from Greiner Bio One (Frickenhausen, Germany). All other chemicals and reagents were of analytical grade.

2.2 CELL CULTURE

The human hepatocellular carcinoma cell line, HepG2, was used in all experiments. Dulbecco's Modified Eagle's Medium (DMEM) liquid medium containing 4500 mg/L dextrose, 4 mg/L pyridoxine hydrogen chloride and 110 mg/L sodium pyruvate was used in cell culture. L-glutamine, penicillin-streptomycin and FBS were added to the media immediately prior to use. HepG2 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM

supplemented with 10% FBS, 2mM L-glutamine, 50 U/ml penicillin and 37.5 U/ml streptomycin (growth medium).

2.2.1 Cell thawing

Cryopreserved HepG2 cells in cryovials were removed from liquid nitrogen and placed into a 37°C water bath until thawed. The cell suspension was transferred to a sterile 15 ml tube and 9 ml of pre-warmed growth medium was slowly added to the cell suspension. Cells were centrifuged at 310 g for 5 min. The supernatant was aspirated and the cells were resuspended in 5 ml of fresh growth medium and transferred into a 25 cm² tissue culture flask (Greiner Bio One, Frickenhausen, Germany). The cells were incubated at 37°C in a 5% CO₂ humidified incubator for 48 h, at which point the medium was replaced.

2.2.2 Passaging cells

After 72 h, cells cultured in 25 cm² tissue culture flasks were 80% confluent. At this time the medium was aspirated and the cells were washed twice with sterile phosphate buffered saline containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.47 mM KH₂PO₄ (pH 7.3) to remove all traces of growth medium. The cells were harvested by adding one ml trypsin-ethylenediaminetetraacetic acid (EDTA) solution, containing 0.25% (w/v) (1: 250) porcine trypsin and 0.02% (w/v) EDTA to the flask and incubating the cells for 5 min at 37°C in a 5% CO₂ humidified incubator. Non-adherent cells were resuspended in 10 ml growth medium, transferred to a sterile 15 ml tube and centrifuged at 310 g for 5 min. The supernatant was aspirated and the cells were resuspended in 5 ml growth medium, transferred to a 75 cm² tissue culture flasks (Greiner Bio One, Frickenhausen, Germany) and placed into a 37°C, 5% CO₂ humidified incubator. This process was repeated several times to produce a working cell bank that contained HepG2 cells

between passage 22 to 28 stored in liquid nitrogen for use in the experiments described in the following chapters. Cells cultured for the cell bank were frozen and stored in liquid nitrogen as described below.

2.2.3 Cell freezing

At 80% confluence, HepG2 cells were harvested from 75 cm² tissue culture flasks using trypsin-EDTA solution, as described in Section 2.2.2, and resuspended in an appropriate volume of freezing medium to yield 5×10^6 cells per ml of freezing medium. The freezing medium contained 70% (v/v) DMEM, 20% (v/v) FBS and 10% (v/v) DMSO. One ml of the cell suspension was transferred into cryogenic vials (Nalgene Nunc International, Naperville, IL, USA) and stored in liquid nitrogen.

2.2.4 Seeding cells

HepG2 cells grown in 75 cm² tissue culture flasks for fatty acid experiments were harvested at 80% confluence. Non-adherent cells were resuspended in 10 ml growth medium, transferred to a sterile 15 ml tube and centrifuged at 310 g for 5 min. The supernatant was aspirated and the cells were resuspended in 5 ml growth medium. To determine the number of cells in suspension, a 50 µL aliquot of the cell suspension was transferred into an eppendorf tube and diluted in 450 µL phosphate buffered saline (PBS). Ten µL of the diluted cell suspension was loaded into a haemocytometer and viewed under a light contrast microscope. The number of cells in four quadrants of the haemocytometer were counted, averaged and multiplied by dilution factors to determine the total number of cells in suspension. This number was used to determine the volume of cell suspension required to seed HepG2 cells into six-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany).

HepG2 cells were seeded into six-well tissue culture plates at a density of 4×10^5 cells/ml in 2 ml of growth medium.

2.2.5 Cell viability

A separate 10 μ L aliquot of the diluted cell suspension was added to 10 μ L 0.4% (w/v) trypan blue solution (trypan blue dye in 0.81% sodium chloride and 0.06% potassium phosphate) (Sigma, St. Louis, MO, USA). Cells with an intact membrane exclude the trypan blue dye and are considered viable. Viable cells and non-viable cells were counted using a haemocytometer. The percent viability was determined by dividing the number of viable cells by the total number of cells. In all experiments described in this thesis, cell viability was determined by trypan blue exclusion.

2.2.6 Supplementation of HepG2 cells with fatty acids

2.2.6.1 Preparation of media

FFA were diluted in analytical grade ethanol to prepare stock solutions at a concentration of 10 mg/ml. Aliquots of each FFA stock solution were stored in 1.5 ml glass vials at -20°C . Fatty acid supplemented serum-free DMEM was prepared immediately prior to use by diluting the FFA stock solution in serum-free DMEM to achieve final concentrations ranging from 0.5-20 $\mu\text{g/ml}$ of the fatty acid. Serum-free media was used since DMEM supplemented with FBS contains fatty acids including DHA (Table 2-1). Moreover, serum free media contains $0.89 \pm 0.04 \mu\text{g/ml}$ of lipid compared with $28.97 \pm 0.73 \mu\text{g/ml}$ in DMEM + 10% FBS.

2.2.6.2 Cell culture

A series of experiments were performed to determine an appropriate protocol for culturing HepG2 cells for investigating the accumulation of fatty acids in cell lipids.

The cells could not be cultured in DMEM +10% FBS for the entirety of experiments involving fatty acid supplementation as DMEM + 10% FBS contained DHA and other fatty acids (Table 2-1). The use of DMEM + 10% charcoal stripped FBS (Sigma, Deisenhofen, Germany) was investigated as charcoal stripped serum contained 26.15 ± 5.25 $\mu\text{g/ml}$ total lipid compared with 169.61 ± 4.30 $\mu\text{g/ml}$ total lipid in standard FBS. However, the number of cells after 6 d culture in DMEM + 10% charcoal stripped FBS were significantly lower (1.9×10^6 cells/ 12.5cm^2) compared with cells maintained in DMEM + 10% FBS ($9.65 \pm 0.74 \times 10^6$ / 12.5cm^2). Cells were seeded and cultured for 3 d in DMEM + 10% FBS to promote growth of the cells and then the media was replaced with either DMEM containing 10% charcoal stripped serum or serum-free DMEM supplemented with 10 $\mu\text{g/ml}$ ALA bound to BSA for 24 h or 48 h. The conditions that produced the greatest accumulation of DHA in cell phospholipids following supplementation with ALA without compromising cell number or viability were considered optimal. The optimal conditions are described below.

For the majority of the experiments described in the following chapters, HepG2 cells were seeded into six-well tissue culture plates and incubated at 37°C in a 5% CO_2 humidified incubator for 72 h. After 72 h, the medium was removed and the cells were washed twice with 1 ml PBS to remove any remaining fatty acid in the medium. Two ml of serum free DMEM supplemented with FFA bound to essentially fatty acid-free BSA was added to each well. The molar ratio of fatty acid to albumin was 4:1. The media was replaced after 24 h with freshly prepared serum-free medium containing the supplemented fatty acid for a further 24 h.

Fatty acid	Serum free	DMEM+10% FBS	Serum free*	DMEM+10% FBS
	μg/ml		% total fatty acids [#]	
Total Saturates	0.34 ± 0.34	11.75 ± 0.26	3.70 ± 3.70	40.59 ± 0.23
18:1n-9	0.55 ± 0.06	7.16 ± 0.22	5.83 ± 0.81	24.72 ± 0.13
Total Monounsaturates	0.55 ± 0.06	10.49 ± 0.31	5.83 ± 0.81	36.19 ± 0.17
20:3n-9	nd	nd	nd	nd
Total n-9	0.55 ± 0.06	7.65 ± 0.25	5.83 ± 0.81	26.39 ± 0.18
18:2n-6	nd	nd	nd	nd
18:3n-6	nd	nd	nd	nd
20:3n-6	nd	0.72 ± 0.05	nd	2.47 ± 0.11
20:4n-6	nd	2.51 ± 0.07	nd	8.67 ± 0.03
22:5n-6	nd	nd	nd	nd
Total n-6		3.23 ± 0.12		11.14 ± 0.13
18:3n-3	nd	nd	nd	nd
20:5n-3	nd	0.11 ± 0.11	nd	0.37 ± 0.37
22:5n-3	nd	1.45 ± 0.03	nd	5.01 ± 0.21
22:6n-3	nd	1.94 ± 0.04	nd	6.70 ± 0.31
Total n-3		3.50 ± 0.07		12.08 ± 0.27
Total Fat μg/ml	0.89 ± 0.40	28.97 ± 0.73		

Table 2-1. Fatty acid composition of serum free DMEM and DMEM with 10% FBS.

Media was extracted for total fatty acid analysis by gas chromatography as described in Materials and Methods. Values are means ± SE. ND not detected, [#] The sum of total fatty acids in serum free DMEM and DMEM + 10% FBS equals 100 with the inclusion of other fatty acids not included in this table. * The remaining 90.5% of the total fatty acids in serum free media was 3-t-butyl-4 hydroxyanisole as described in Section 2.3.3.

2.2.7 Cell harvest

After 48 h incubation with fatty acid supplemented medium, the cells were harvested using trypsin-EDTA solution. Fifty μL of trypsin-EDTA solution was added to each well and the six-well tissue culture plate was incubated at 37°C in a 5% CO_2 , humidified incubator for 5 min. Cells from three wells were collected and pooled in 1.5 ml PBS and transferred into an eppendorf tube. Fifty μL of the cell suspension was used to determine cell number and viability as described above (see Section 2.2.4). The cell suspension was transferred to a glass tube for fatty acid analysis (see Section 2.3).

2.3 FATTY ACID ANALYSIS

2.3.1 Lipid analysis of HepG2 cells

Lipids were extracted from cells, suspended in PBS, with chloroform-methanol (2:1, by vol) according to the method of Folch *et al.* (1957) (171). Two ml methanol and 80 μL C17:0 (phospholipid, triglyceride, cholesterol ester, 1:1:1 by vol) as internal standard were added to the cell suspension and mixed well, followed by the addition of 4 ml chloroform. The glass extraction tube was shaken vigorously and allowed to stand for 5 min at room temperature. Samples were centrifuged at 1500 g for 10 min to separate the aqueous and organic phase. The organic phase containing lipid, was extracted and transferred to a glass scintillation vial and evaporated under a stream of nitrogen. The lipid extract was reconstituted in 150 μL chloroform: methanol (9:1, by vol) and spotted onto 0.3 mm Silica Gel (60H) thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany). Lipid fractions were separated by thin layer chromatography (TLC) using a mobile-phase of petroleum spirits, diethyl ether and glacial acetic acid (180:30:2, by vol) and visualised under ultraviolet light.

Phospholipid, triglyceride and cholesterol ester fractions were identified by comparison to a TLC reference standard (Nu-Check-Prep Inc., Elysian, MN, USA) and collected into separate 2 ml glass vials containing 2 ml 1 % sulphuric acid in methanol (v/v) with 0.005% butylated hydroxyanisole (BHA) (w/v) as antioxidant. The lipid fractions were methylated at 70°C for 3 h. The resulting FAME were extracted into 250 µL distilled water and 500 µL n-heptane. The upper heptane layer containing the FAME was removed and transferred to gas chromatography vials containing anhydrous sodium sulphate. The samples were concentrated under a stream of nitrogen to a final volume of 100 µL for analysis by gas chromatography.

2.3.2 Fatty acid analysis of media

Fatty acid-supplemented media was analysed to compare the actual concentration of the supplemented fatty acid in the medium with the theoretical concentration. The fatty acid composition of DMEM + 10% FBS and serum free DMEM was also analysed (Table 2-1). A 1 ml aliquot of medium was transferred to a glass extraction tube and weighed before the addition of 0.5 ml cold isotonic saline (Baxter, Deerfield, IL, USA), 2 ml of methanol and 80 µL of C17:0 FFA (Sigma, St Louis, MO, USA) as internal standard. The sample was mixed well and 4 ml of chloroform was added. The glass extraction tube was shaken vigorously and allowed to stand for 5 min at room temperature. Samples were centrifuged at 1500 g for 10 min to separate the aqueous and organic phase. The chloroform layer, containing lipid, was extracted and transferred to a glass scintillation vial and evaporated under nitrogen. The lipid extract was reconstituted in 5 ml 1 % sulphuric acid in methanol (v/v) with 0.005% BHA (w/v) as antioxidant. Total lipids were methylated at 70°C for 2 h. The resulting FAME were extracted into 750 µL distilled water and 2 ml n-heptane. The upper heptane layer containing the FAME was removed and transferred to gas

chromatography vials containing anhydrous Na₂SO₄ and evaporated under nitrogen to approximately 600 µL for analysis by gas chromatography.

2.3.3 Analysis of FAME by gas chromatography

FAME were measured by gas chromatography on a BPX-70 50 m capillary column coated with 70% cyanopropyl polysilphenylene-siloxane (0.25 µm film thickness and 0.32 mm internal diameter SGE, Victoria, Australia) on a Hewlett-Packard 6890 gas chromatograph (GC) (Hewlett Packard, Palo Alto, CA, USA) fitted with a flame ionisation detector. Helium was the carrier gas and the split-ratio was 20:1. The injection port temperature was 250°C and the detector temperature was 300°C. The initial column temperature was 140°C and increased to 220°C at a rate of 5°C/min and held at 220°C for up to 3 min. The identity of each fatty acid peak was ascertained by comparison of peak retention time to an authentic lipid standard (NuChek Prep, MN, USA) (Figure 2-1). The standard contained 52 FAME however, the GC program only measured 48 of these FAME of relevance to the samples being analysed. The relative amount of each fatty acid (% of total fatty acid) was quantified by integrating the area under the peak and dividing the result by the total area for all fatty acids. A typical trace of the GC analysis of FAME derived from HepG2 cell phospholipids is shown in

Figure 2-2. The limit of detection was 0.05% total fatty acids.

In the gas chromatographic analysis of media, a peak eluted at the same retention time as LA. This was analysed by gas chromatography-mass spectrometry (GC-MS) and identified as 3-t-butyl-4-hydroxyanisole. This appeared to be characteristic of the cell culture media as peaks that eluted at this retention time in lipid extracts from HepG2 cells were identified as the FAME of LA by GC-MS. The fatty acid

composition of media is summarised in Table 2-1. The majority of fatty acids accumulated in the phospholipid fraction of HepG2 cell lipid extracts, accounting for approximately 66% of the total fat content. Minimal quantities accumulated in the cholesterol ester fraction and approximately 33% accumulated in triglycerides (Table 2-2). The fatty acid composition of HepG2 cell phospholipids following supplementation with fatty acids is reported in this thesis given that this fraction represented the greatest accumulation of fatty acids in HepG2 cell lipids.

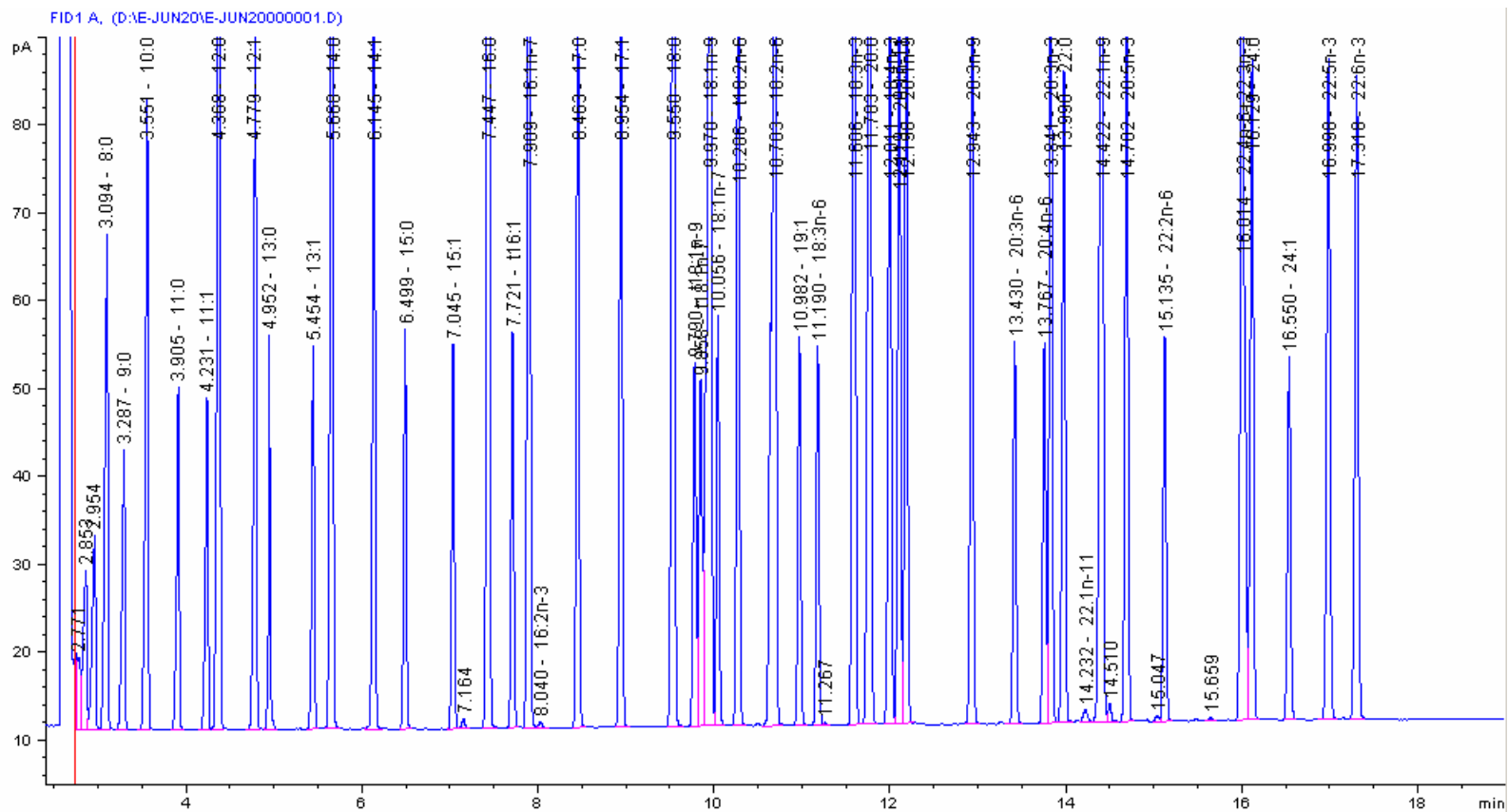


Figure 2-1. Gas chromatogram of authentic lipid standards (NuChek Prep, MN, USA).

The retention time of each fatty acid peak in the standard was used to compare and identify peaks eluted from samples.

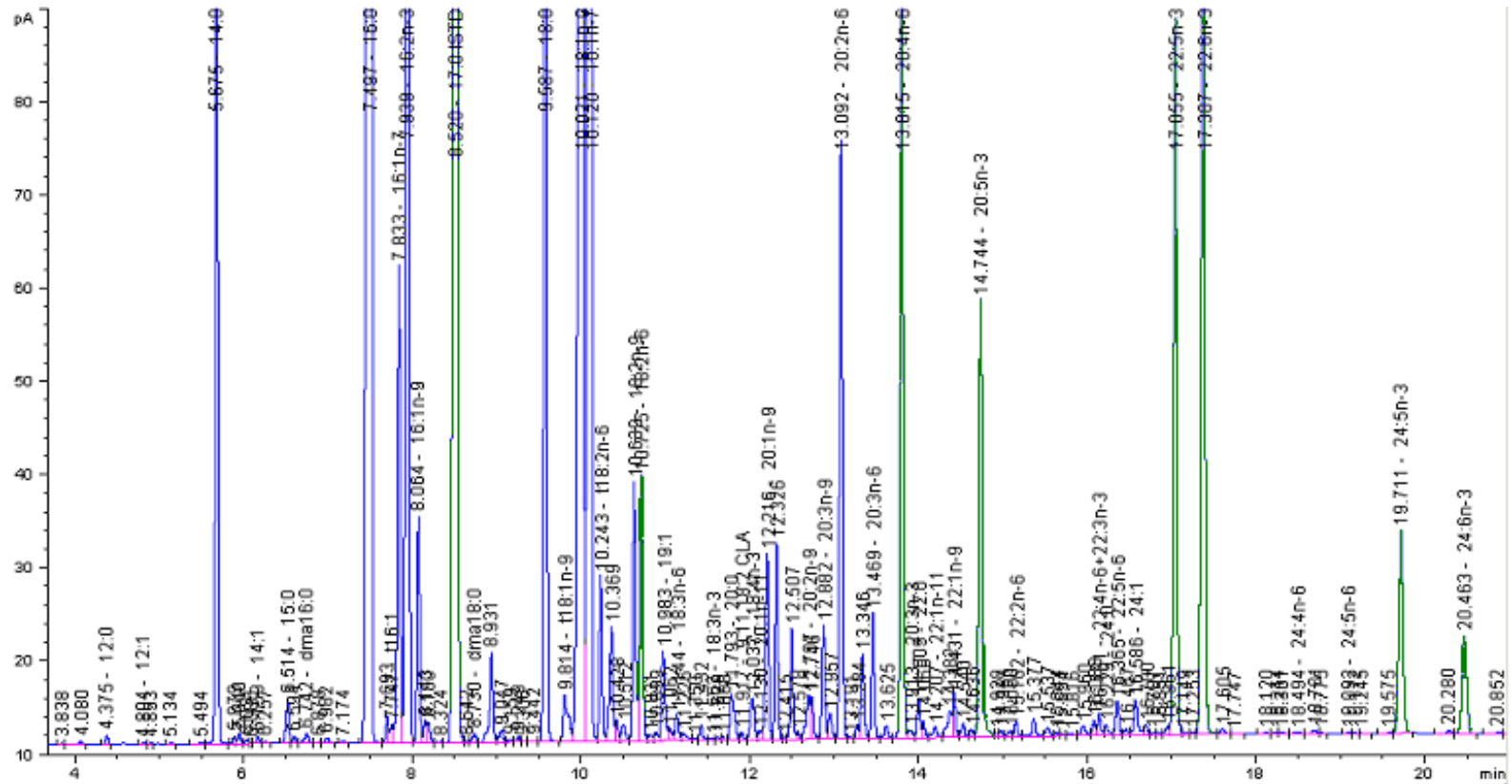


Figure 2-2. A typical gas chromatogram of fatty acid methyl esters derived from HepG2 cell phospholipids.

HepG2 cells were supplemented with 10 $\mu\text{g/ml}$ docosapentaenoic acid (22:5n-3) for 48 h and then harvested for fatty acid analysis, as described in Materials and Methods.

Lipid fraction	Phospholipids	Triglycerides	Cholesterol esters
	μg/million cells		
Fatty acid			
Total Saturates	12.75 ± 0.52	6.00 ± 0.82	0.16 ± 0.02
18:1n-9	5.35 ± 0.26	4.66 ± 0.50	0.29 ± 0.01
Total Monounsaturates	16.51 ± 0.97	11.29 ± 1.12	0.57 ± 0.02
20:3n-9	0.39 ± 0.24	0.02 ± 0.01	nd
Total n-9	7.80 ± 0.33	5.48 ± 0.59	0.32 ± 0.02
18:2n-6	0.34 ± 0.05	0.07 ± 0.04	0.09 ± 0.01
18:3n-6	0.01 ± 0.01	0.03 ± 0.01	nd
20:3n-6	0.15 ± 0.01	0.01 ± 0.004	nd
20:4n-6	0.97 ± 0.03	0.02 ± 0.004	nd
22:5n-6	0.05 ± 0.003		nd
Total n-6	2.36 ± 0.28	0.18 ± 0.05	0.09 ± 0.01
18:3n-3	0.70 ± 0.08	0.37 ± 0.11	0.04 ± 0.001
20:5n-3	1.73 ± 0.05	0.04 ± 0.01	nd
22:5n-3	0.44 ± 0.03	0.10 ± 0.02	nd
22:6n-3	1.66 ± 0.04	0.13 ± 0.02	nd
Total n-3	5.04 ± 0.15	1.05 ± 0.26	0.09 ± 0.02
Total Fat μg/million cells	38.14 ± 1.66	18.90 ± 2.09	0.95 ± 0.06

Table 2-2. Concentration of fatty acids in phospholipid, triglyceride and cholesterol ester fractions of HepG2 cells supplemented with serum-free medium containing 5 μg/ml ALA. ND not detected

2.4 IDENTIFICATION OF 24 CARBON FATTY ACIDS IN HEPG2 CELL LIPID FRACTIONS

In HepG2 cells supplemented with ALA, EPA and DPA, two peaks beyond the retention time of DHA were apparent in the GC analysis of FAME in the phospholipid fraction (Figure 2-3A). These peaks were suspected to be the chain elongation product of DPA, 24:5n-3 and the Δ 6 desaturation product of 24:5n-3, 24:6n-3. Commercial standards for 24:5n-3 and 24:6n-3 were not available, however, these fatty acids occur in the jellyfish *Aurelia* sp. (172). FAME from the phospholipid fraction of HepG2 cells supplemented with DPA were sent to laboratories at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Marine Research, Hobart, Tasmania, Australia for analysis by gas chromatography. At the laboratories, the retention times of these unknown peaks were compared with FAME derived from the *Aurelia* sp. GC analysis of the FAME from HepG2 cells at CSIRO Marine Research Laboratories identified two peaks that had comparable retention times to 24:5n-3 and 24:6n-3 in the jelly fish *Aurelia* sp. The mass spectra of these unknown peaks were also examined.

2.4.1 Gas Chromatography-Mass Spectrometry analysis

FAME were analysed by GC-MS on a Hewlett Packard 6890 GC (Palo Alto, CA, USA) equipped with a BPX-70 50 m capillary column (0.25 μ m film thickness and 0.22 mm internal diameter SGE, Victoria, Australia), and a 5972 mass selective detector. Helium was the carrier gas at a flow of 1.9 ml/min. For analysis, 2 μ L volumes were injected into a split/splitless injector set at 250°C with a split ratio of 20:1. The initial column temperature was 140°C and increased to 220°C at a rate of 5°C/min, which was held at 220°C for 2 min. A second heat ramp to 260°C

(20°C/min) was performed and maintained at 260°C for 8 min. A 70 eV ionisation potential was applied and the mass spectrometry acquisition of each sample was set to SCAN mode.

GC-MS analysis of these FAME also eluted two unknown peaks at 20.01 min and 20.34 min (Figure 2-3A). The fragment pattern for the first unknown peak was (major ions at m/z) 79, 91, 108, 119, 133, 147, 161 and 186 corresponding to 24:5n-3 as described in the jellyfish *Aurelia* sp. (172) (Figure 2-3B) and also contained the characteristic ion at m/z 108, typical of fatty acids with an n-3 terminal group (173). The mass spectra of the second unknown peak contained 79, 91, 105, 119, 128, 137, 147, 157 and 193 corresponding to 24:6n-3 as described in the jellyfish *Aurelia* sp. (172)(Figure 2-3C). The mass spectra and retention times of these peaks confirmed that they were 24:5n-3 and 24:6n-3, respectively. The homologous n-6 FAME were also identified in the phospholipid fraction of HepG2 cells supplemented with docosatetraenoic acid (DTA, 22:4n-6). Analysis by GC-MS identified a major ion at m/z 150, characteristic of fatty acids with an n-6 terminal moiety (173). The retention time and mass spectra of these peaks indicated that they were 24:4n-6 and 24:5n-6.

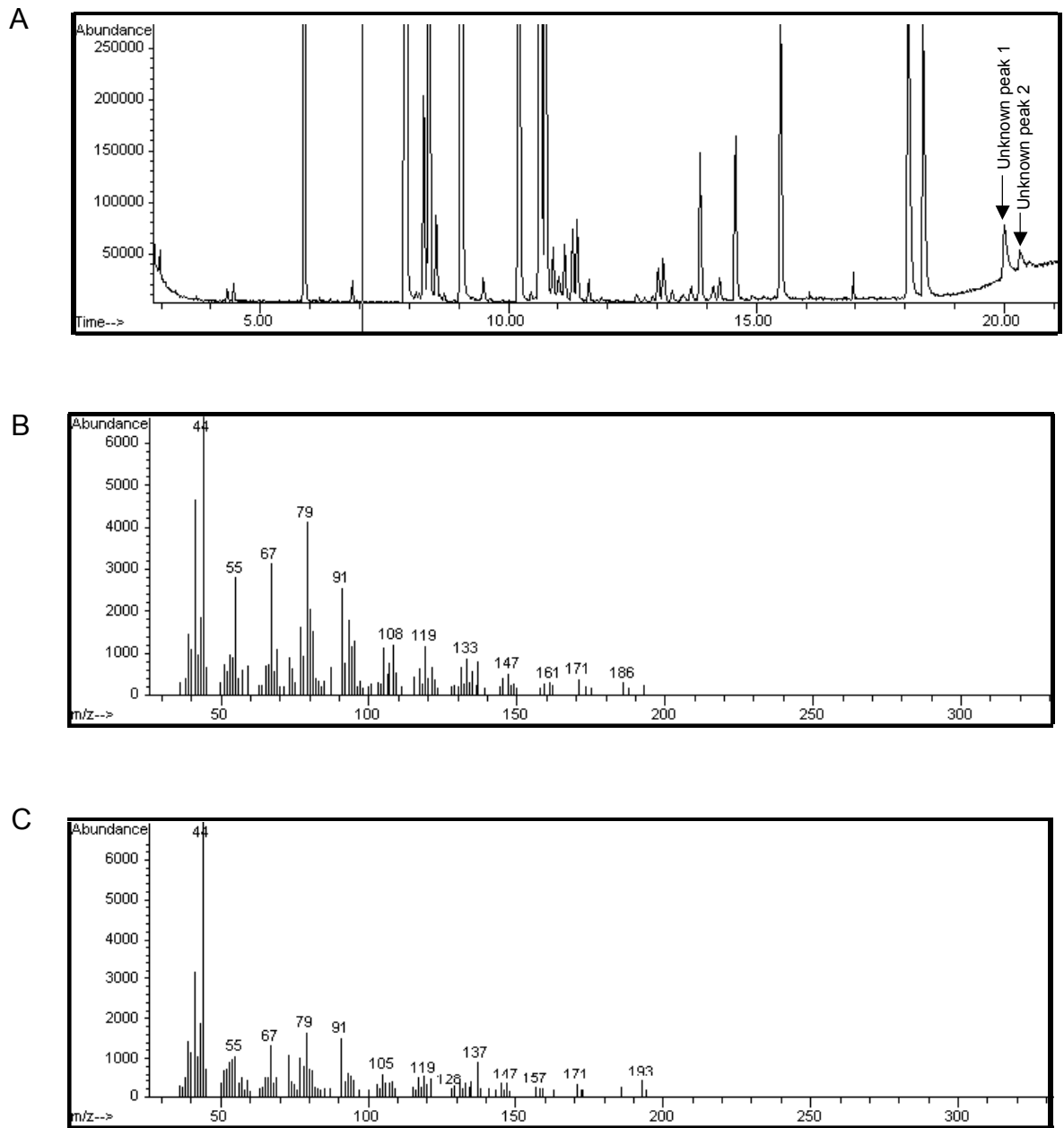


Figure 2-3. Gas Chromatography-Mass Spectrometry analysis of unknown fatty acid methyl esters in the phospholipid fraction of lipid extracted from HepG2 cells supplemented with 10 $\mu\text{g}/\text{ml}$ docosapentaenoic acid.

(A) Gas chromatogram of HepG2 cell phospholipid fraction containing two unknown peaks at 20.01 min and 20.34 min respectively. (B) Mass spectra of unknown peak 1, subsequently identified as 24:5n-3. (C) Mass spectra of unknown peak 2, subsequently identified as 24:6n-3.

2.5 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) and Bonferroni post hoc statistical tests (SPSS Inc Chicago, IL, USA) were used to compare means following fatty acid supplementation on means of at least three separate replicates. Statistical significance was defined as $p < 0.05$. Data are expressed as mean \pm standard error (SE).

3 Conversion of ALA and LA in HepG2 cells

3.1 INTRODUCTION

In vivo dietary intervention studies indicate a limitation in the accumulation of DHA following supplementation with ALA (7;30-32;174). This observation may be partly explained by the intake of dietary LA, which is typically 10-fold higher than the intake of ALA in most populations (65;175;176). As described in Chapter 1, ALA and LA utilise common enzymes in their conversion to LCPUFA. Therefore dietary LA will compete with dietary ALA for enzymes involved in the synthesis of DHA from ALA. The reported rate-limiting enzyme in the synthesis of DHA from ALA is D6D, with ALA allegedly being the preferred substrate (96). The affinity of D6D for ALA is approximately two to three times that of LA (113) yet the dietary intake of LA is generally greater. The competition for D6D also extends to the conversion of the 24 carbon fatty acids (24:5n-3 and 24:4n-6) to DHA and 22:5n-6, respectively. The extent to which the competition between n-3 and n-6 fatty acids for common enzymes contributes to the poor accumulation of DHA from ALA described in human supplementation trials is unclear. The use of D6D twice in the synthesis of DHA from ALA suggests that ALA itself may also be a key regulator of accumulation of DHA from ALA.

This chapter aimed to examine the accumulation of LCPUFA in HepG2 cell phospholipids following supplementation with ALA or LA individually and exclusively of each other. The fatty acid composition of HepG2 cell phospholipids following fatty acid supplementation was compared with the accumulation of n-3 and n-6 fatty acids in tissues *in vivo* to establish the use of this human cell line as a model of dietary supplementation *in vivo*. The dynamics of fatty acid uptake and

accumulation were also examined by measuring the accumulation of ALA and its metabolites in HepG2 cell phospholipids over 48 h.

3.2 MATERIALS AND METHODS

The materials and methods used were those described in Chapter 2, with the following changes.

3.2.1 Cell culture

HepG2 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin and 37.5 U/ml streptomycin. Cells were grown in 75 cm² tissue culture flasks and incubated at 37°C in a 5% CO₂ humidified incubator. At 80% confluence, HepG2 cells were harvested and seeded into six-well plates (Greiner Bio One, Frickenhausen, Germany) as described in Section 2.2.4. After 72 h, the medium was removed and replaced with serum-free DMEM supplemented with increasing amounts of ALA or LA bound to fatty acid-free BSA. Stock solutions of these fatty acids were prepared in ethanol at a concentration of 10 mg/ml and diluted in serum-free medium to achieve final concentrations ranging from 0.5-20 µg/ml ALA or LA. Cells were harvested after 48 h incubation with the fatty acid. To measure the accumulation of fatty acids over time, HepG2 cells were cultured in DMEM with 10% FBS for 72 h. The media was then replaced with serum free DMEM supplemented with 20 µg/ml ALA bound to BSA. Cells were harvested at time zero, then at 1, 2, 6, 12, 24 and 48 h after supplementation with ALA. The fatty acid composition of HepG2 cells maintained in serum-free DMEM, supplemented with the equivalent concentration of BSA, was measured at each time as the control.

3.2.2 Fatty acid analysis

Total lipids were extracted from cells following procedures described in Chapter 2.

3.2.3 Statistical analysis

Fatty acid analyses were performed on means of at least three separate replicates as described in Section 2.5. Where appropriate, regression analysis was used to fit curves of best fit to the data (SPSS SigmaPlot Inc Chicago, IL, USA).

3.3 RESULTS

3.3.1 Accumulation of n-3 fatty acids

The accumulation of ALA into cell phospholipids was concentration-dependent, increasing from $0.12 \pm 0.11\%$ to $7.05 \pm 0.53\%$ total fatty acids over the concentration range tested ($0.5\text{-}20 \mu\text{g/ml}$ ALA) (Figure 3-1). The accumulation of EPA and DPA was also dependent on the concentration of ALA in the media (Figure 3-1). The level of EPA in cell phospholipids increased 47-fold from $0.15 \pm 0.02\%$ to $7.07 \pm 0.74\%$ total fatty acids and the level of DPA increased 7-fold, from $0.25 \pm 0.05\%$ to $1.91 \pm 0.31\%$ total fatty acids. In contrast, the level of DHA in cell phospholipids peaked following supplementation with $5 \mu\text{g/ml}$ ALA and did not increase with further increases in the concentration of ALA. At its peak, the level of DHA in cell phospholipids had increased 1.7-fold from $2.62 \pm 0.06\%$ to $4.44 \pm 0.33\%$ total fatty acids.

The accumulation of $24:5n-3$ and $24:6n-3$ in cell phospholipids was also measured. There was a dose-dependent relationship between the accumulation of the D6D substrate, $24:5n-3$ in cell phospholipids and the concentration of ALA in the media (Figure 3-2). The accumulation of the product of D6D, $24:6n-3$, however, paralleled the accumulation of DHA. There was an increase in the level of $24:6n-3$, up to 5

µg/ml ALA supplementation but no increase with increased concentrations of ALA (Figure 3-2).

The fatty acid composition of HepG2 cell phospholipids following supplementation with increasing concentrations of ALA are summarised in Table 3-1. The level of saturated fatty acids increased concomitant with a decrease in the total level of monounsaturated fatty acids in HepG2 cell phospholipids following supplementation with increasing concentrations of ALA, suggesting an exchange in the distribution of fatty acids with ALA-supplementation. The total level of n-9 fatty acids decreased with increasing concentration of ALA. However, with ALA supplementation, the level of n-6 fatty acids did not change.

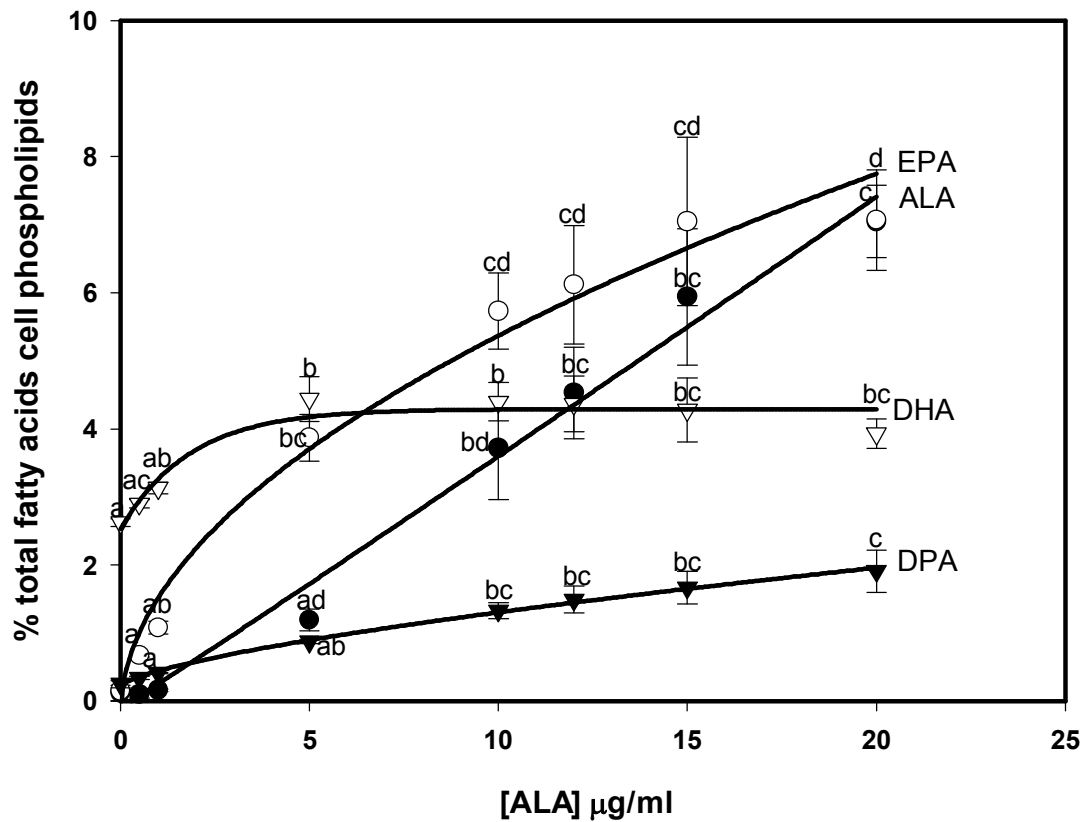


Figure 3-1. The accumulation of α -linolenic acid (18:3n-3, ALA) (●), eicosapentaenoic acid (20:5n-3, EPA) (○), docosapentaenoic acid n-3 (22:5n-3, DPA) (▼), and docosahexaenoic acid (22:6n-3, DHA) (▽) in HepG2 cell phospholipids of cells supplemented with ALA.

HepG2 cells were cultured as described and supplemented with increasing concentrations of ALA for 48 h. Values are means \pm SE of at least three independent experiments with three replicates in each experiment. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group. Regression curves have been fitted to the data.

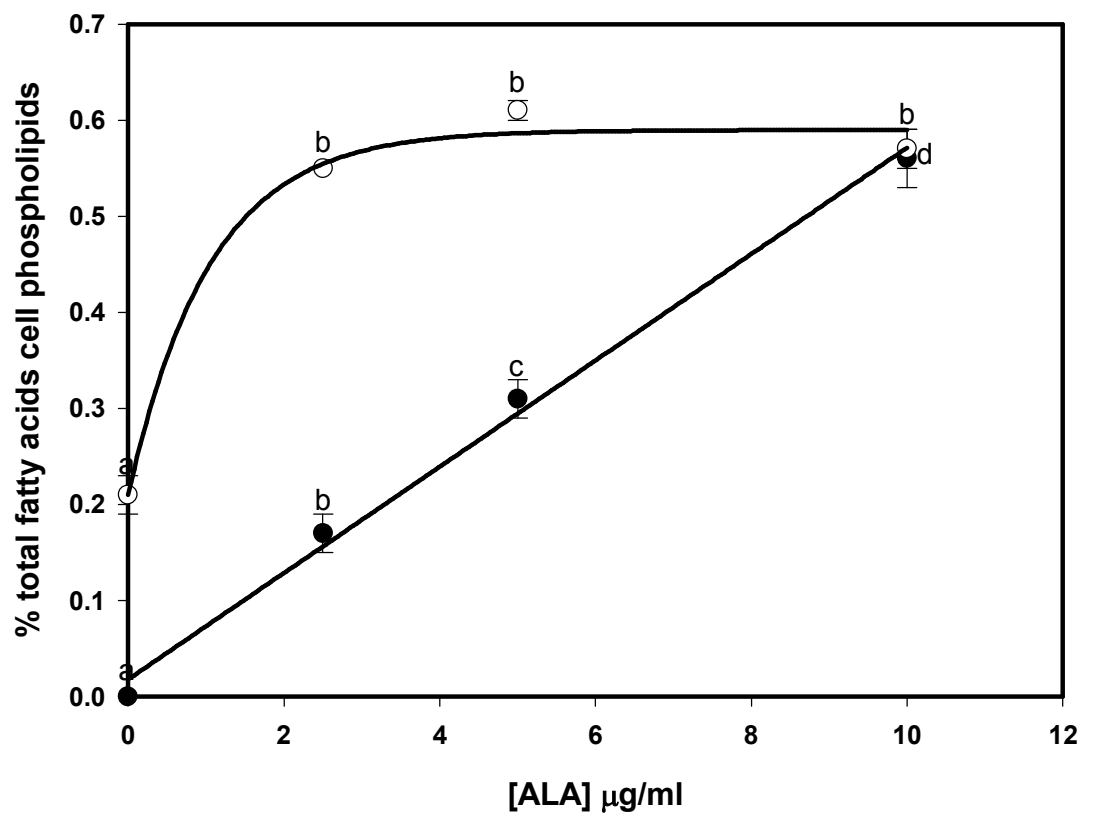


Figure 3-2 The accumulation of 24:5n-3 (●) and 24:6n-3 (○) in HepG2 cell phospholipids following supplemented with α -linolenic acid (18:3n-3, ALA).

HepG2 cells were cultured as described and supplemented with increasing concentrations of ALA for 48 h. Values are means \pm SE of at least three independent experiments with three replicates in each experiment. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group. Regression curves have been fitted to the data.

	BSA control	0.5µg/ml ALA	1µg/ml ALA	5µg/ml ALA	10µg/ml ALA	12µg/ml ALA	15µg/ml ALA	20µg/ml ALA
Fatty acid	% total fatty acids							
Total Saturates	29.69 ± 0.20 ^a	30.06 ± 0.38 ^a	31.27 ± 0.60 ^{a,b}	33.18 ± 0.18 ^{a,b,c}	35.12 ± 0.54 ^{b,c}	34.94 ± 0.90 ^{b,c}	35.33 ± 0.77 ^{b,c}	35.29 ± 1.44 ^c
18:1n-9 OA	20.72 ± 0.48 ^a	19.53 ± 0.25 ^{a,b}	18.95 ± 1.09 ^{a,b}	16.57 ± 0.49 ^{b,c}	13.98 ± 0.64 ^{c,d}	13.33 ± 0.82 ^{c,d}	11.85 ± 0.98 ^d	11.23 ± 0.75 ^d
Total Monounsaturates	51.81 ± 0.20 ^a	51.02 ± 0.42 ^a	49.43 ± 1.20 ^a	44.74 ± 0.26 ^b	38.44 ± 1.31 ^c	36.80 ± 1.40 ^{c,d}	33.76 ± 1.94 ^{c,d}	32.34 ± 1.60 ^d
20:3n-9	5.30 ± 0.31 ^a	5.21 ± 0.29 ^a	4.85 ± 0.16 ^{a,b}	3.50 ± 0.23 ^{b,c}	2.63 ± 0.30 ^c	2.69 ± 0.30 ^c	2.35 ± 0.26 ^c	2.24 ± 0.21 ^c
Total n-9	33.15 ± 0.25 ^a	31.82 ± 0.33 ^a	30.70 ± 0.53 ^a	25.11 ± 0.65 ^b	20.64 ± 0.95 ^c	20.08 ± 1.29 ^c	18.21 ± 1.37 ^c	17.56 ± 1.20 ^c
18:2n-6 LA	0.74 ± 0.05 ^{a,b}	0.67 ± 0.01 ^a	0.73 ± 0.06 ^{a,b}	0.70 ± 0.02 ^{a,b}	0.83 ± 0.03 ^{a,b}	0.83 ± 0.03 ^{a,b}	0.92 ± 0.08 ^b	0.86 ± 0.01 ^{a,b}
18:3n-6	0.08 ± 0.03 ^a	0.11 ± 0.01 ^a	0.10 ± 0.01 ^a	0.10 ± 0.003 ^a	0.09 ± 0.01 ^a	0.09 ± 0.003 ^a	0.09 ± 0.02 ^a	0.09 ± 0.02 ^a
20:3n-6	0.49 ± 0.02 ^a	0.47 ± 0.02 ^{a,b}	0.47 ± 0.02 ^{a,b}	0.45 ± 0.01 ^{a,b}	0.44 ± 0.01 ^{a,b}	0.45 ± 0.01 ^{a,b}	0.43 ± 0.01 ^{a,b}	0.43 ± 0.01 ^b
20:4n-6 AA	2.99 ± 0.12 ^a	2.87 ± 0.04 ^a	2.84 ± 0.07 ^a	2.69 ± 0.04 ^a	2.78 ± 0.13 ^a	2.95 ± 0.24 ^a	2.94 ± 0.34 ^a	2.64 ± 0.08 ^a
22:5n-6 DPAn-6	0.14 ± 0.04 ^a	0.13 ± 0.05 ^a	0.12 ± 0.04 ^a	0.12 ± 0.01 ^a	0.13 ± 0.01 ^a	0.15 ± 0.02 ^a	0.16 ± 0.03 ^a	0.14 ± 0.03 ^a
Total n-6	4.93 ± 0.22 ^a	4.84 ± 0.30 ^a	4.81 ± 0.20 ^a	4.46 ± 0.21 ^a	4.54 ± 0.16 ^a	4.72 ± 0.24 ^a	4.76 ± 0.37 ^a	4.40 ± 0.13 ^a
18:3n-3 ALA	0.12 ± 0.11 ^a	0.10 ± 0.06 ^a	0.16 ± 0.02 ^a	1.19 ± 0.16 ^{a,d}	3.72 ± 0.76 ^{b,d}	4.53 ± 0.67 ^{b,c}	5.94 ± 1.00 ^{b,c}	7.05 ± 0.53 ^c
20:5n-3 EPA	0.15 ± 0.02 ^a	0.67 ± 0.02 ^a	1.08 ± 0.09 ^{a,b}	3.87 ± 0.34 ^{b,c}	5.73 ± 0.56 ^{c,d}	6.12 ± 0.87 ^{c,d}	7.05 ± 1.24 ^{c,d}	7.07 ± 0.74 ^d
22:5n-3 DPAn-3	0.25 ± 0.05 ^a	0.34 ± 0.02 ^a	0.41 ± 0.01 ^a	0.87 ± 0.03 ^{a,b}	1.33 ± 0.12 ^{b,c}	1.49 ± 0.20 ^{b,c}	1.67 ± 0.24 ^{b,c}	1.91 ± 0.31 ^c
22:6n-3 DHA	2.62 ± 0.06 ^a	2.90 ± 0.06 ^{a,c}	3.13 ± 0.08 ^{a,b}	4.44 ± 0.33 ^b	4.40 ± 0.28 ^b	4.37 ± 0.41 ^b	4.28 ± 0.47 ^{b,c}	3.93 ± 0.22 ^{b,c}
Total n-3	3.99 ± 0.24 ^a	4.80 ± 0.08 ^a	5.59 ± 0.23 ^{a,b}	11.44 ± 0.59 ^{b,c}	16.81 ± 1.27 ^{c,d}	18.42 ± 1.77 ^{a,e}	21.13 ± 2.10 ^{a,e}	22.69 ± 1.44 ^e

Table 3-1. Fatty acid composition of HepG2 cell phospholipids of cells supplemented with α -linolenic acid (18:3n-3, ALA).

HepG2 cells were cultured as described and supplemented with increasing concentrations of ALA for 48 h. Values are means \pm SE of at least three replicates. Values with different superscripts are significantly different from each other ($p < 0.05$) by Bonferroni post-hoc test. ($p < 0.05$).

3.3.2 Accumulation of preformed DHA

To examine whether the limited accumulation of DHA from ALA was due to a limitation in its incorporation into cell phospholipids, the accumulation of DHA derived from ALA was compared with the accumulation of DHA when it was supplemented preformed in the media. DHA readily accumulated in HepG2 cell phospholipids when it was supplied in the media, increasing from $3.14 \pm 0.27\%$ in control cells to $16.26 \pm 0.61\%$ total fatty acids in cells supplemented with $10 \mu\text{g/ml}$ DHA (Figure 3-3). The accumulation of DHA in cell phospholipids when supplied directly in the media was also curvilinear and appears to approach a plateau. The fatty acid composition of HepG2 cell phospholipids following supplementation with increasing concentrations of DHA are summarised in Table 3-2. The total level of saturated fatty acids in cell phospholipids increased following supplementation with DHA. The total level of monounsaturated fatty acids and n-9 fatty acids decreased with increasing concentrations of DHA. The level of EPA in HepG2 cell phospholipids increased linearly with increasing concentrations of DHA. The total level of n-6 fatty acids in cell phospholipids was unchanged by the supplementation of HepG2 cells with DHA.

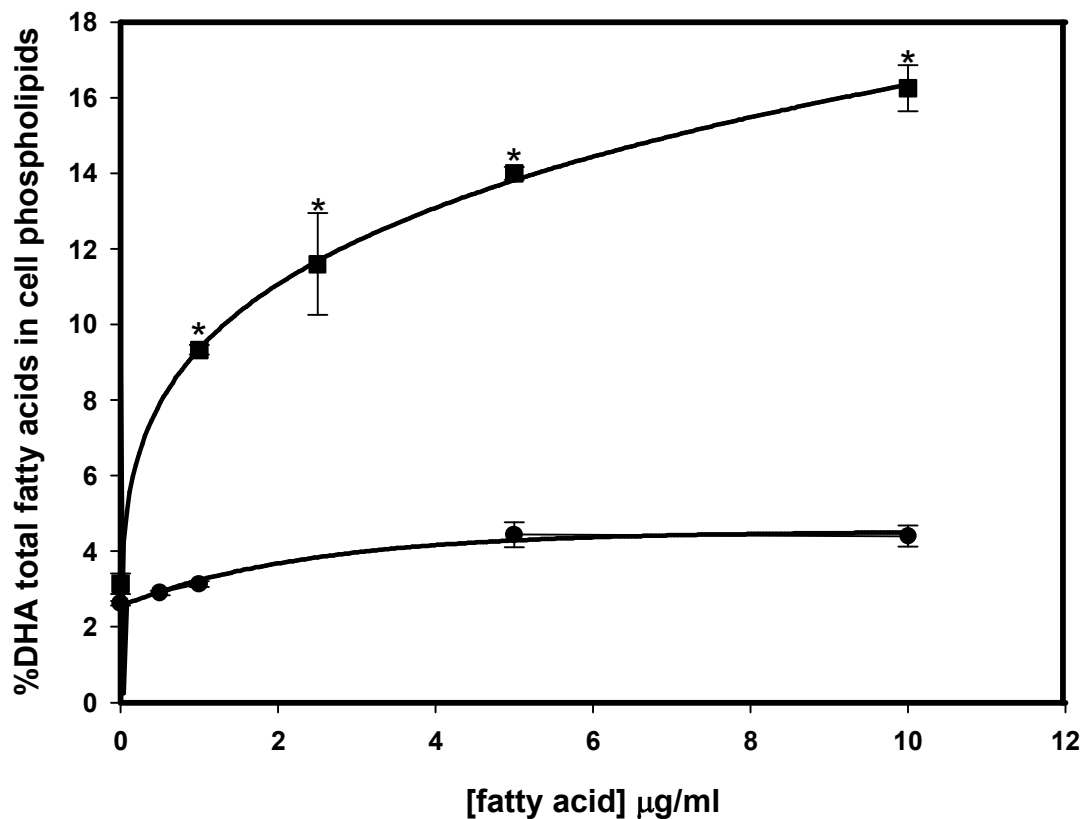


Figure 3-3. The accumulation of docosahexaenoic acid (22:6n-3, DHA) in HepG2 cell phospholipids following supplemented with α -linolenic acid (18:3n-3, ALA) (●) or preformed DHA (■).

HepG2 cells were cultured as described and supplemented with increasing concentrations of ALA for 48 h. Values are means \pm SE of at least three independent experiments with three replicates in each experiment. Values marked with an asterisk were significantly different ($p < 0.05$) between supplementation groups. Regression curves have been fitted to the data.

Fatty acid	BSA control		1 µg/ml DHA		2.5 µg/ml DHA		5 µg/ml DHA		10 µg/ml DHA	
	% total fatty acids									
Total Saturates	29.13	± 0.13 ^a	30.73	± 0.13 ^a	32.33	± 0.65 ^{a, b}	34.91	± 0.93 ^{b, c}	37.29	± 1.01 ^c
18:1n-9 OA	19.50	± 0.84 ^a	17.57	± 0.03 ^{a, b}	16.70	± 0.92 ^{a, b}	14.88	± 0.71 ^{c, d}	12.78	± 0.32 ^d
Total Monounsaturates	52.15	± 0.79 ^a	46.46	± 0.25 ^{a, b}	43.35	± 2.30 ^{b, c}	38.05	± 2.19 ^{c, d}	32.71	± 1.63 ^d
20:3n-9	4.56	± 0.35 ^a	3.24	± 0.04 ^{a, b}	3.08	± 0.41 ^b	2.57	± 0.21 ^b	2.12	± 0.08 ^b
Total n-9	29.71	± 1.69 ^a	24.96	± 0.09 ^{a, b}	24.10	± 2.02 ^{a, b}	21.17	± 1.44 ^b	18.02	± 0.78 ^b
18:2n-6 LA	1.43	± 0.31 ^a	1.81	± 0.01 ^a	1.72	± 0.48 ^a	2.40	± 0.81 ^a	3.34	± 1.26 ^a
18:3n-6	0.11	± 0.03 ^a	0.06	± 0.05 ^a	0.12	± 0.002 ^a	0.04	± 0.04 ^a	0.03	± 0.03 ^a
20:3n-6	0.63	± 0.07 ^a	0.76	± 0.01 ^a	0.68	± 0.09 ^a	0.76	± 0.13 ^a	0.79	± 0.12 ^a
20:4n-6 AA	3.39	± 0.28 ^a	3.64	± 0.05 ^a	3.24	± 0.35 ^a	3.44	± 0.46 ^a	3.42	± 0.40 ^a
22:5n-6 DPA n-6	0.18	± 0.08 ^a	0.22	± 0.002 ^a	0.11	± 0.05 ^a	0.025	± 0.00 ^a	0.025	± 0.00 ^a
Total n-6	5.92	± 0.77 ^a	6.48	± 0.10 ^a	5.89	± 0.94 ^a	6.65	± 1.34 ^a	7.61	± 1.71 ^a
18:3n-3 ALA	0.13	± 0.10 ^a	0.025	± 0.00 ^a	0.01	± 0.01 ^a	0.025	± 0.00 ^a	0.025	± 0.00 ^a
20:5n-3 EPA	0.18	± 0.06 ^a	0.26	± 0.001 ^a	0.53	± 0.17 ^{a, b}	1.20	± 0.29 ^b	2.11	± 0.15 ^c
22:5n-3 DPA n-3	0.33	± 0.06 ^a	0.29	± 0.02 ^a	0.26	± 0.03 ^a	0.36	± 0.05 ^a	0.37	± 0.07 ^a
22:6n-3 DHA	3.14	± 0.27 ^a	9.33	± 0.10 ^b	11.60	± 1.34 ^{b, c}	14.00	± 0.17 ^{c, d}	16.26	± 0.61 ^d
Total n-3	5.15	± 0.68 ^a	11.03	± 0.06 ^b	13.23	± 1.59 ^c	16.10	± 0.46 ^{c, d}	19.15	± 0.78 ^d

Table 3-2. Fatty acid composition of HepG2 cell phospholipids of cells supplemented with preformed docosahexaenoic acid (22:6n-3, DHA). Values are means ± SE of at least three replicates. HepG2 cells were cultured as described and supplemented with increasing concentrations of DHA for 48 h. Values with different superscripts are significantly different from each other ($p < 0.05$) by Bonferroni post-hoc test. ($p < 0.05$).

3.3.3 Accumulation of n-3 fatty acids over a 48 hour time-course

The change in fatty acid composition of HepG2 cell phospholipids over the 48 h supplementation period was also investigated. The maximum accumulation of ALA in HepG2 cell phospholipids occurred at 6 h, increasing from $0.29 \pm 0.18\%$ total fatty acids prior to supplementation with ALA to $8.97 \pm 0.39\%$ total fatty acids at 6 h (Figure 3-4). After 48 h, the level of ALA had equilibrated to $6.26 \pm 0.52\%$ total fatty acids. The rate of accumulation of ALA calculated from the area-under-the-curve was 279.40% total fatty acid•h. The level of EPA increased steadily over the 48 h supplementation period. The rate of accumulation of EPA calculated from the area-under-the-curve was 258.14% total fatty acid•h. The level of DHA remained relatively unchanged (Figure 3-4). However, Figure 3-5 shows that the level of DHA in HepG2 cells maintained in serum-free DMEM gradually decreased over 48 h. The level of DHA in HepG2 cell phospholipids immediately prior to supplementation with ALA was $4.26 \pm 0.58\%$ total fatty acids, which was attributed to the availability of DHA in DMEM + 10% FBS, containing $6.70 \pm 0.31\%$ DHA total fatty acids. Incubation of HepG2 cells with $20\ \mu\text{g/ml}$ ALA maintained the initial level of DHA so that there was a net increase in the level of DHA over the 48 h supplementation period compared with control cells, indicating synthesis. The rate of accumulation of DHA and DPA calculated from the area-under-the-curve was 207.70% total fatty acid•h and 63.90% total fatty acid•h, respectively.

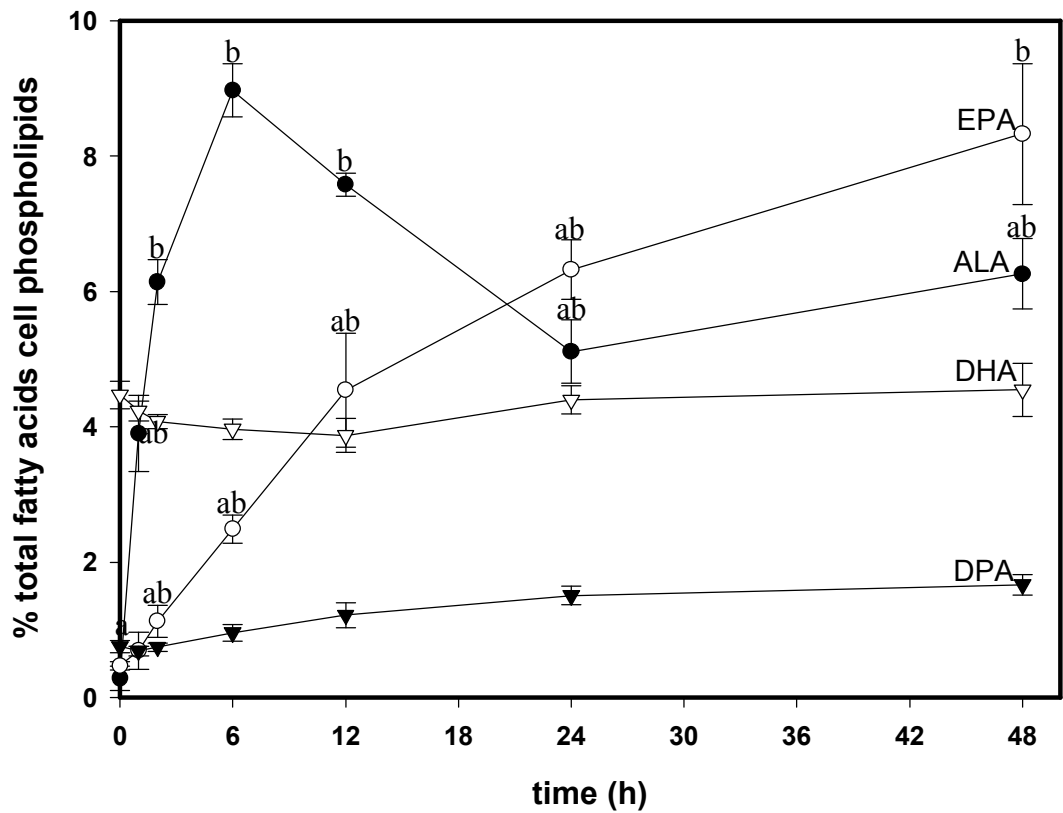


Figure 3-4. The accumulation of α -linolenic acid (18:3n-3, ALA) (●), eicosapentaenoic acid (20:5n-3, EPA) (○), docosapentaenoic acid n-3 (22:5n-3, DPA) (▼), and docosahexaenoic acid (22:6n-3, DHA) (▽) in HepG2 cell phospholipids of cells supplemented with 20 μ g/ml ALA over 48 h. HepG2 cells were cultured as described and supplemented with 20 μ g/ml ALA and harvested at the times indicated to 48 h. Values are means \pm SE of at least three independent experiments with three replicates in each experiment. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group. There was no significant difference in the level of DPA or DHA between each time point.

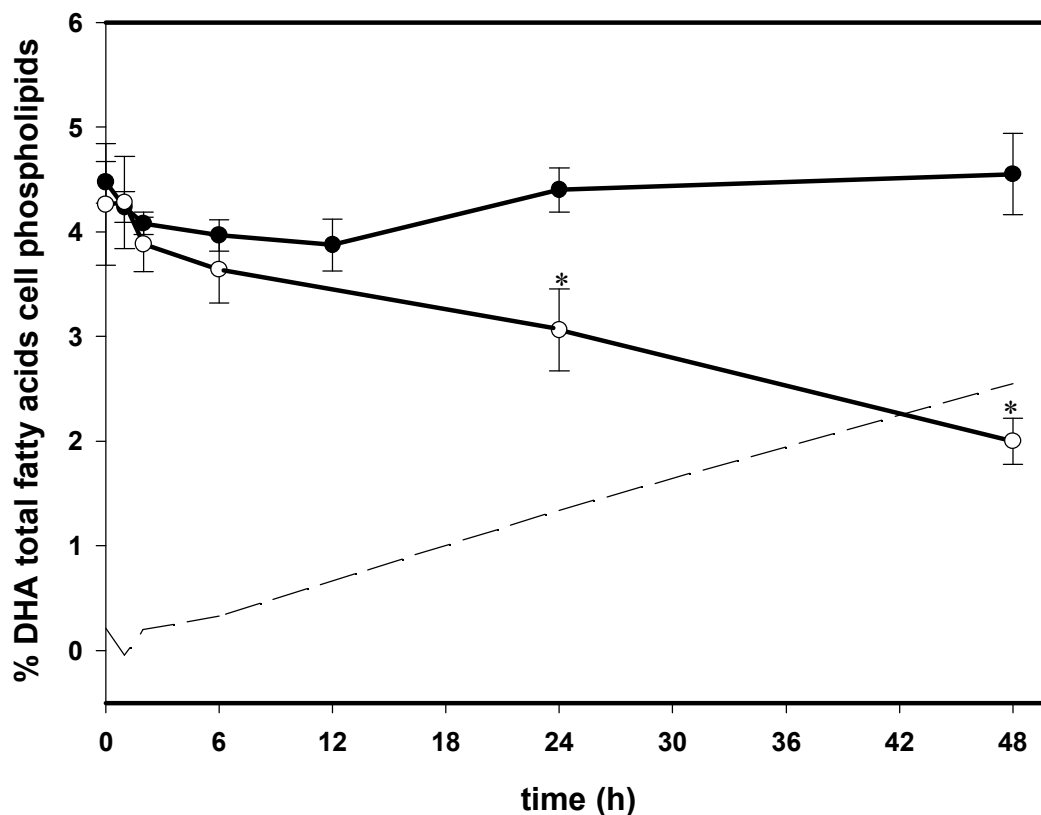


Figure 3-5. The content of docosahexaenoic acid in cell phospholipids of HepG2 cells incubated in serum free DMEM supplemented with 20 $\mu\text{g/ml}$ α -linolenic acid (18:3n-3, ALA), bound to BSA, (●), or serum free DMEM with BSA alone (○) and the net change in the level of docosahexaenoic acid (22:6n-3, DHA) (dashed line). HepG2 cells were cultured as described and supplemented with 20 $\mu\text{g/ml}$ ALA and harvested at the times indicated to 48 h. Values are means \pm SE of at least three independent experiments with three replicates in each experiment. Values marked with an asterisk are significantly different from cells supplemented with ALA by Independent Student's T-test ($p < 0.05$).

3.3.4 Accumulation of n-6 fatty acids

The fatty acid composition of HepG2 cells supplemented with the n-6 essential fatty acid, LA was also examined. The fatty acid composition of HepG2 cell phospholipids following supplementation with increasing concentrations of LA are summarised in Table 3-3. The accumulation of LA into cell phospholipids was concentration-dependent as was the accumulation of AA (Figure 3-6). After supplementation with 20 µg/ml LA, the level of LA in cell phospholipids increased 20-fold from $0.73 \pm 0.06\%$ to $15.92 \pm 0.24\%$ total fatty acids. The level of AA increased 3.6-fold from $2.94 \pm 0.14\%$ to $10.6 \pm 0.83\%$ total fatty acids and DPA n-6 (22:5n-6) increased 7-fold from $0.17 \pm 0.01\%$ to $1.19 \pm 0.17\%$ total fatty acids. The D6D product of LA, GLA (18:3n-6), did not accumulate in significant amounts. The level of saturated fatty acids increased concomitant with a decrease in the total level of monounsaturated fatty acids in HepG2 cell phospholipids following supplementation with increasing concentrations of LA, suggesting an exchange in the distribution of fatty acids following LA-supplementation. Under conditions of LA supplementation, the total level of n-3 fatty acids did not change.

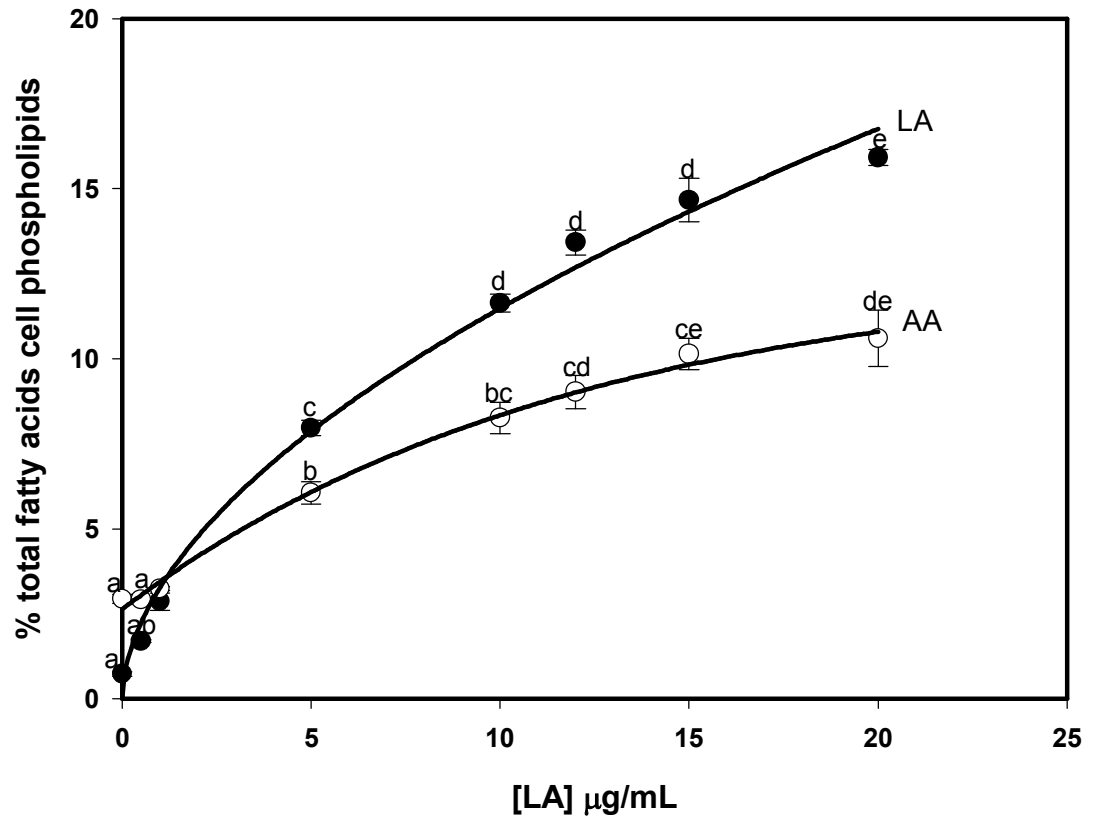


Figure 3-6. The accumulation of linoleic acid (18:2n-6, LA) (●) and arachidonic acid (20:4n-5, AA) (○) in HepG2 cell phospholipids of cells supplemented with LA.

HepG2 cells were cultured as described and supplemented with increasing concentrations of LA for 48 h. Values are means \pm SE of at least three independent experiments with three replicates in each experiment. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group. Regression curves have been fitted to the data.

	BSA control	0.5µg/ml LA	1µg/ml LA	5µg/ml LA	10µg/ml LA	12µg/ml LA	15µg/ml LA	20µg/ml LA
Fatty acid	% total fatty acids							
Total Saturates	29.63 ± 0.18 ^a	29.68 ± 0.10 ^a	29.92 ± 0.06 ^a	32.37 ± 0.45 ^b	33.92 ± 0.33 ^{b,c}	33.45 ± 0.25 ^{b,c}	33.80 ± 0.45 ^{b,c}	34.55 ± 0.30 ^c
18:1n-9 OA	20.69 ± 0.52 ^a	19.20 ± 0.47 ^a	18.75 ± 0.52 ^a	15.33 ± 0.82 ^b	12.76 ± 0.59 ^{b,c}	12.15 ± 0.65 ^{b,c}	10.89 ± 0.68 ^c	9.86 ± 0.87 ^c
Total Monounsaturates	51.99 ± 0.27 ^a	51.03 ± 0.45 ^a	49.32 ± 0.41 ^a	40.99 ± 1.49 ^b	34.46 ± 1.18 ^c	31.69 ± 0.83 ^{c,d}	28.88 ± 0.74 ^{d,e}	26.92 ± 1.67 ^e
20:3n-9	5.36 ± 0.36 ^a	5.58 ± 0.41 ^a	5.38 ± 0.31 ^{a,c}	2.86 ± 1.26 ^{a,b}	2.15 ± 0.89 ^b	2.67 ± 0.07 ^{c,d}	2.28 ± 0.07 ^{c,d}	2.12 ± 0.19 ^d
Total n-9	33.19 ± 0.31 ^a	31.97 ± 0.64 ^a	30.98 ± 0.74 ^a	23.39 ± 0.47 ^b	18.97 ± 0.55 ^c	18.51 ± 0.58 ^{c,d}	16.67 ± 0.86 ^{c,d}	15.02 ± 1.22 ^d
18:2n-6 LA	0.73 ± 0.06 ^a	1.70 ± 0.03 ^{a,b}	2.86 ± 0.26 ^b	7.97 ± 0.23 ^c	11.64 ± 0.26 ^d	13.42 ± 0.37 ^d	14.67 ± 0.64 ^d	15.92 ± 0.24 ^e
18:3n-6	0.11 ± 0.03 ^a	0.12 ± 0.06 ^{a,b}	0.26 ± 0.01 ^b	0.43 ± 0.02 ^c	0.44 ± 0.02 ^c	0.44 ± 0.01 ^c	0.44 ± 0.02 ^c	0.43 ± 0.01 ^c
20:3n-6	0.48 ± 0.02 ^a	0.65 ± 0.02 ^a	0.91 ± 0.04 ^a	2.27 ± 0.12 ^b	2.75 ± 0.06 ^b	2.73 ± 0.13 ^b	2.83 ± 0.22 ^b	2.83 ± 0.20 ^b
20:4n-6 AA	2.94 ± 0.14 ^a	2.92 ± 0.01 ^a	3.24 ± 0.06 ^a	6.06 ± 0.33 ^b	8.26 ± 0.46 ^{b,c}	9.03 ± 0.49 ^{c,d}	10.14 ± 0.47 ^{c,e}	10.60 ± 0.83 ^{d,e}
22:5n-6 DPA n-6	0.17 ± 0.01	0.19 ± 0.01	0.21 ± 0.00	0.40 ± 0.20	0.61 ± 0.31	0.73 ± 0.31	0.87 ± 0.38	1.19 ± 0.17
Total n-6	4.78 ± 0.28 ^a	5.90 ± 0.31 ^a	7.83 ± 0.59 ^a	17.65 ± 0.66 ^b	24.37 ± 0.58 ^c	27.26 ± 0.61 ^{c,d}	30.09 ± 0.61 ^{d,e}	32.22 ± 1.70 ^e
18:3n-3 ALA	0.11 ± 0.13 ^a	0.025 ± 0.00 ^a	0.025 ± 0.00 ^a	0.025 ± 0.00 ^a	0.025 ± 0.00 ^a	0.025 ± 0.00 ^a	0.22 ± 0.22 ^a	0.025 ± 0.00 ^a
20:5n-3 EPA	0.14 ± 0.02	0.11 ± 0.03	0.13 ± 0.03	0.11 ± 0.02	0.10 ± 0.01	0.06 ± 0.01	0.05 ± 0.02	0.07 ± 0.01
22:5n-3 DPA n-3	0.24 ± 0.06	0.18 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.20 ± 0.04	0.25 ± 0.09	0.17 ± 0.01
22:6n-3 DHA	2.61 ± 0.08 ^a	2.53 ± 0.06 ^a	2.56 ± 0.01 ^a	2.47 ± 0.01 ^a	2.29 ± 0.07 ^{a,b}	2.40 ± 0.16 ^{a,b}	1.88 ± 0.28 ^b	2.11 ± 0.06 ^{a,b}
Total n-3	3.95 ± 0.29	3.70 ± 0.07	3.64 ± 0.08	3.45 ± 0.04	3.17 ± 0.06	3.14 ± 0.16	2.99 ± 0.13	2.75 ± 0.07

Table 3-3. Fatty acid composition of HepG2 cell phospholipids of cells supplemented with linoleic acid (18:2n-6, LA).

HepG2 cells were cultured as described and supplemented with increasing concentrations of LA for 48 h. Values are means ± SE of at least three replicates. Values with different superscripts are significantly different from each other (p < 0.05) by Bonferroni post-hoc test. (p < 0.05).

3.3.5 Cell number and viability

Supplementation of HepG2 cells with increasing concentrations of ALA or LA, bound to BSA, did not affect cell number or viability compared with control cells ($p > 0.05$) (Figure 3-7). The number of cells increased over the 48 h supplementation period, almost doubling from the beginning of supplementation to the end at 48 h (Figure 3-8). Cell viability did not change over the 48 h supplementation period with either fatty acid (Figure 3-8).

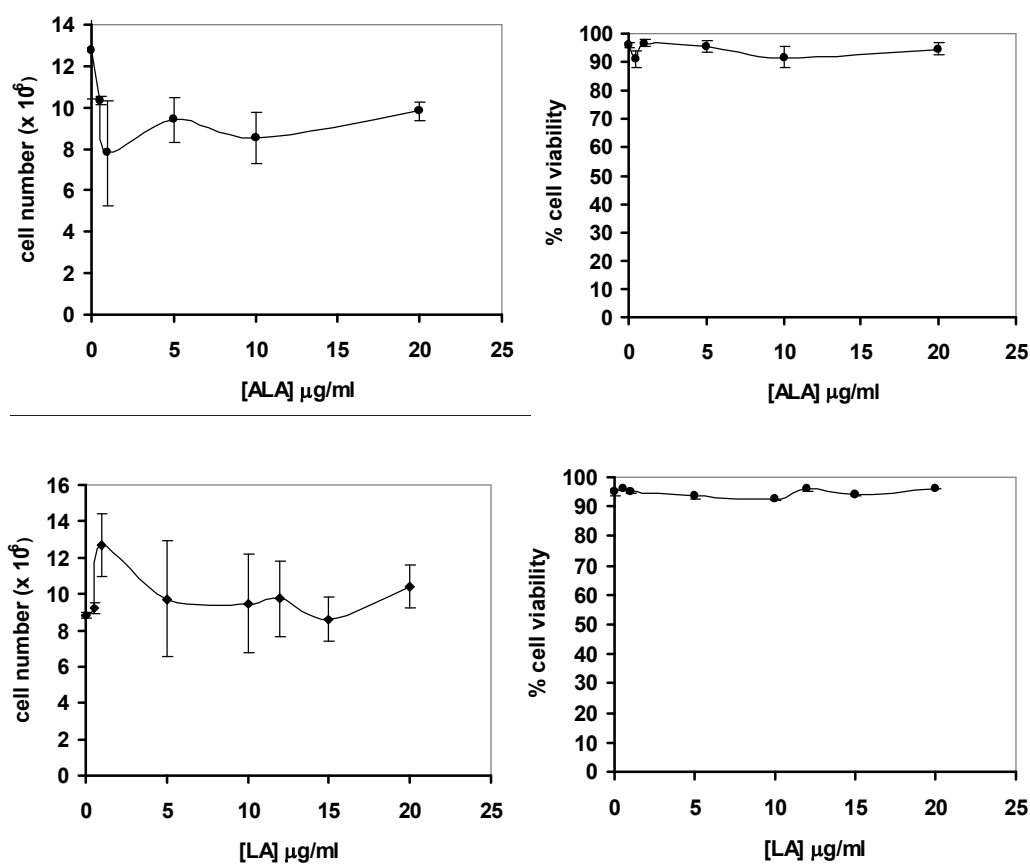


Figure 3-7. Cell number and viability of HepG2 cells supplemented with increasing concentrations of α -linolenic acid (18:3n-3, ALA) or linoleic acid (18:2n-6, LA).

HepG2 cells were incubated for 48 h with increasing concentrations of ALA or LA bound to BSA in serum-free DMEM. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.

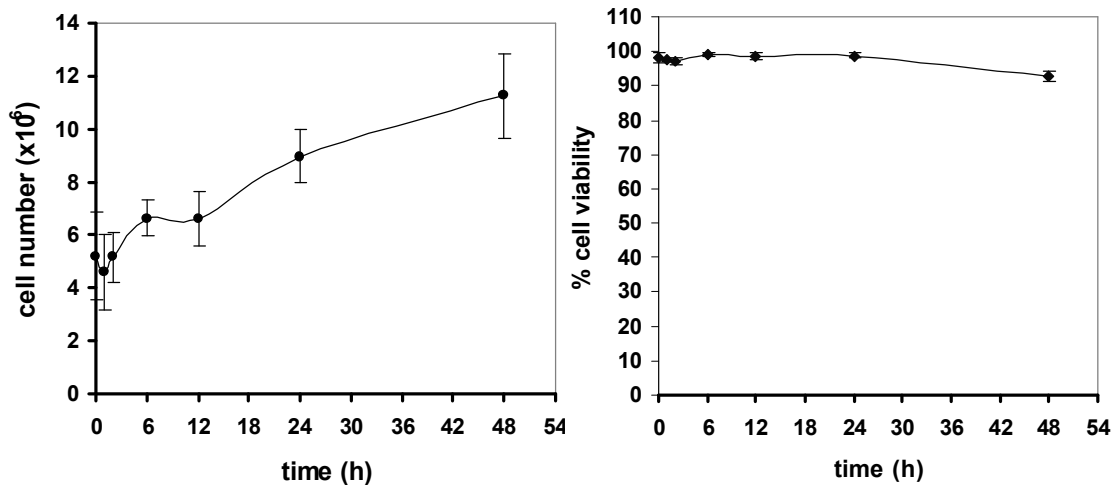


Figure 3-8. Cell number and viability of HepG2 cells supplemented with 20 $\mu\text{g/ml}$ α -linolenic acid (18:3n-3, ALA) over a 48 h time-course.

HepG2 cells were incubated for with 20 $\mu\text{g/ml}$ ALA bound to BSA in serum-free DMEM. Cells were harvested immediately prior to supplementation (t=0), then at 1, 2, 6, 12, 24 and 48 h supplementation. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.

3.4 DISCUSSION

The pattern of accumulation of LCPUFA following supplementation with ALA and LA in HepG2 cells described here supports the proposed pathway of fatty acid conversion (93-95) and indicates that the HepG2 cell system is a suitable model for examining the conversion and accumulation of fatty acids into cell lipids. Other cell lines including human mammary carcinoma cells, ZR-75-1 (158), and human colon carcinoma cells, CaCo-2 (159), have been used to demonstrate the capacity of *in vitro* cell systems to mimic *in vivo* fatty acid conversion using labelled fatty acids. However, this is the first study to thoroughly describe mass fatty acid compositional changes in HepG2 cell phospholipids following supplementation with ALA and LA.

The fatty acid composition of cell phospholipids following supplementation, a net effect of fatty acid conversion and incorporation into cell phospholipids, was measured in order to evaluate the efficacy of ALA and LA in altering membrane fatty acid composition and to compare the accumulation of fatty acids with that described *in vivo* (6;7;30;157). The limited accumulation of DHA from ALA described here in HepG2 cells is consistent with that described *in vivo*. A dose-response relationship between ALA and the level of EPA in cell phospholipids, concomitant with a limitation in the accumulation of DHA with increasing concentration of ALA, was observed in HepG2 cells. The linear accumulation of EPA in cells supplemented with increasing concentrations of ALA suggests that the conversion of ALA to EPA, which involves D6D and D5D, may not be the limiting steps in the accumulation of DHA from ALA. The dose-dependent accumulation of DPA and 24:5n-3 in cells supplemented with ALA also suggests that the enzymes involved in the elongation of fatty acids and incorporation into cell phospholipids was not limiting. However, the curvilinear accumulation of the D6D products,

24:6n-3 and DHA, implicates D6D and events following the $\Delta 6$ desaturation of 24:5n-3 in regulating the accumulation of DHA. The data in Figure 3-2 suggests that the limited accumulation of DHA in cell phospholipids following supplementation with ALA may be due to competition between ALA and 24:5n-3 for active sites on D6D. D6D is used twice in the synthesis of DHA from ALA. With increasing concentrations of ALA, the $\Delta 6$ desaturation of 24:5n-3 to 24:6n-3 may be competitively inhibited. Because D6D has a higher affinity for ALA than 24:5n-3 (95), it is reasonable to suggest that the $\Delta 6$ desaturation of 24:5n-3 is inhibited with increasing concentrations of ALA, preventing the synthesis of DHA from ALA.

The level of DHA in cell phospholipids when supplied preformed was significantly elevated, compared with cells supplemented with ALA and suggests that HepG2 cells have the capacity to incorporate up to 16% total fatty acids as DHA into cell phospholipids. The increased incorporation of DHA may be due to an increased pool of DHA available for acylation into cell phospholipids compared with that available from the conversion of ALA. However, the accumulation of preformed DHA in cell phospholipids was curvilinear and suggests that acylation of DHA into cell phospholipids and/or trafficking of fatty acid between organelles may also be important regulatory steps in the accumulation of DHA (177;178).

There are clear parallels between the accumulation of n-3 and n-6 fatty acids in HepG2 cells when compared to an *in vivo* system as demonstrated by an early study by two pioneers in the field, Mohrhauer and Holman (1963) (134). By examining the total fatty acid composition of the liver of rats maintained on a fat-free diet supplemented with increasing amounts of pure ALA or LA as the only source of dietary fat they demonstrated a dose-dependent increase in the level of ALA in the

livers of supplemented rats, with an accompanying increase in the level of EPA and DPA (134). The level of DHA in liver lipids increased dramatically when ALA provided up to 1% calories. Further increases in percent calories of ALA, in the absence of competing n-6 substrates, did not significantly elevate the accumulation of DHA in liver lipids (134) and mirrors the pattern of accumulation of DHA reported here in HepG2 cells. The pattern of accumulation of n-6 fatty acids *in vitro* (Figure 3-6) also paralleled that described in rats maintained on a fat free diet supplemented with increasing calories of pure LA (134). Mohrhauer and Holman (1963) report a dose-dependent increase in the level of LA in the livers of supplemented rats, with an accompanying increase in the level AA (134). The pattern of n-3 and n-6 fatty acid accumulation in rats and HepG2 cells are remarkably similar and provides further support for the use of this cell line as a model of fatty acid conversion *in vivo*.

The competition between ALA and 24:5n-3 for D6D inferred from the pattern of accumulation of fatty acids reported here in HepG2 cells may explain the limited accumulation of DHA in tissues following ALA-supplementation *in vivo*. In a meta-regression analysis of plasma phospholipid n-3 fatty acid concentration following ALA supplementation in humans, a linear dose-response relationship was reported for ALA and EPA, however the concentration of DHA was unaffected by ALA supplementation of up to 14 g/d (179). In animals fed increasing amounts of ALA, a proportional enrichment of EPA in tissues has been reported but, counter-intuitively, the level of DHA was reduced in animals fed the highest amount of ALA (136;139).

The direct relationship between ALA-supplementation and the level of EPA in cell membranes and the poor accumulation of DHA following supplementation with

ALA was also evident in HepG2 cells. Even in the absence of competing n-6 substrates, a limitation in the accumulation of DHA following supplementation with increasing amounts of ALA was observed in HepG2 cell phospholipids. The regulation of DHA synthesis by ALA is surprising and indicates that dietary interventions using ALA-rich dietary fats may not be useful in elevating the level of DHA in tissues even when the dietary intake of LA is minimal.

There are marked parallels between the *in vivo* and *in vitro* metabolism of ALA yet the metabolism of LA between the two models displays some significant differences. There appears to be a greater level of regulation in the accumulation of AA from LA *in vivo* compared with the level of regulation *in vitro*. Conversion of LA to AA involves $\Delta 6$ desaturation of LA followed by chain elongation to 20:3n-6 and then $\Delta 5$ desaturation to yield AA. An almost five-fold increase the dietary intake of LA in marmosets reduced the level of AA in erythrocyte phospholipids by 20% (180). Concomitant with this reduction was an increase in the level of LA in erythrocyte phospholipids suggesting that LA competes with AA for incorporation into tissue phospholipids. One study has shown however, that increased dietary intakes of LA elevated the level of AA in plasma phospholipids by 48% (140). Many of animal studies are complicated by the presence of other fatty acids in the diet, particularly ALA (140;180). Discrepancies among animal studies may be explained by a critical dose of LA at which point the synthesis and accumulation of AA is maximal. Lands *et al.* (1990) (135) developed an empirical equation from rat feeding studies and showed a hyperbolic relationship between tissue AA and the intake of LA ranging from 0 to 0.33 en%. A dietary intake of LA greater than 0.33 en% resulted in no further increase in the concentration of AA (135). It may be possible that the concentrations used in the experiments performed here in HepG2 cells were below

the point at which the conversion of LA to AA becomes saturated. It is more likely that the discrepancy between the accumulation of AA from LA in HepG2 cell phospholipids with that reported *in vivo* results from competition between LA and ALA for D6D since D6D has a higher affinity for ALA (96).

In conclusion, the limited accumulation of DHA from ALA reported here is consistent with the concept that the synthesis of DHA from ALA is tightly regulated. The linear accumulation of EPA in HepG2 cells following supplementation with increasing concentrations of ALA indicates that the conversion of ALA is not limited at the first $\Delta 6$ desaturation step. The accumulation of the immediate secondary D6D substrate, 24:5n-3, was also linear and suggests that the elongation of DPA to 24:5n-3 was not limiting. In contrast, the accumulation of the post D6D product, 24:6n-3 in cell phospholipids was curvilinear and paralleled the accumulation of DHA following supplementation with increasing concentrations of ALA. The utilisation of D6D by both 24:5n-3 and ALA infers that competition between 24:5n-3 and ALA for active sites on D6D will directly influence the capacity for cell membranes to accumulate DHA in significant amounts. The incorporation of DHA into cell phospholipids will also influence the accumulation of DHA in cell membranes. The pattern of accumulation of n-3 LCPUFA in HepG2 cells supplemented with ALA implicates the competition between ALA and 24:5n-3 as a major point of regulation in the synthesis of DHA from ALA and may explain why ALA intervention trials in animals and humans have been ineffective at increasing tissue levels of DHA.

4 Metabolism of LCPUFA in HepG2 cells

4.1 INTRODUCTION

The final enzymatic steps in the conversion of DPA to DHA had been controversial until it was demonstrated that the same D6D acts on both 18 carbon fatty acids and 24 carbon fatty acids (95). DPA is elongated to 24:5n-3, which is desaturated by D6D in the endoplasmic reticulum to yield 24:6n-3, this is then translocated to the peroxisome where two carbons are removed by β -oxidation to yield DHA (95). In Chapter 3, a limited accumulation of DHA from ALA in HepG2 cell membranes was observed and may be explained by the competition between ALA and 24:5n-3 for D6D with increasing concentrations of ALA. In the absence of ALA, there is only one D6D substrate involved in the synthesis of DHA from EPA or DPA, that is, 24:5n-3. When EPA or DPA is the only available fatty acid *in vitro*, the Δ 6 desaturation of 24:5n-3 is not competitively inhibited by ALA. This suggests that supplementation with EPA or DPA may enhance the accumulation of DHA in cell membranes by bypassing the first Δ 6 desaturation step required in the synthesis of DHA from ALA. It was hypothesised that the accumulation of DHA in HepG2 cells supplemented with EPA or DPA would be greater than the accumulation from ALA due to a reduced D6D requirement.

This chapter examines the accumulation of n-3 LCPUFA in cells supplemented with EPA or DPA. The accumulation of n-6 LCPUFA was also examined in cells supplemented with docosatetraenoic acid (DTA, 22:4n-6, is the n-6 homologue of DPA) to demonstrate the parallels between the accumulation of n-3 and n-6 fatty acids. The accumulation of preformed DHA in HepG2 cell phospholipids is also presented (as in Chapter 3). To determine whether competition for incorporation into

cell phospholipids between fatty acids contributes significantly to the limited accumulation of DHA from n-3 fatty acid precursors, the accumulation of DHA in cell phospholipids in the presence of other fatty acids was examined.

4.2 MATERIALS AND METHODS

The materials and methods used were those described in Chapter 2, with the following changes.

4.2.1 Cell culture

HepG2 cells were cultured and seeded into six-well plates as described in Section 3.2.1. After 72 h, the medium was removed and replaced with serum-free DMEM supplemented with EPA, DPA, DTA or DHA bound to fatty acid-free BSA. Stock solutions of these fatty acids were prepared in ethanol at a concentration of 10 mg/ml and diluted in serum-free medium to achieve final concentrations ranging from 1-10 µg/ml EPA, DPA, DTA or DHA. Cells were harvested after 48 h supplementation with the fatty acid (described in Section 2.2.7). In experiments aimed to examine the competition between fatty acids for incorporation into cell phospholipids, HepG2 cells were supplemented with serum-free medium containing 5 µg/ml DHA, bound to BSA, alone or in addition to 1 - 10 µg/ml ALA, LA or EPA. Cells were harvested, as described previously (Section 2.2.7) after 2 h supplementation. The supplementation time was reduced to limit the conversion of the supplemented fatty acids to long chain metabolites.

4.2.2 Fatty acid analysis

Lipids were extracted from cells following procedures described in Chapter 2.

4.2.3 Statistical analysis

Fatty acid analyses were performed on means of at least three separate replicates as described in Section 2.5.

4.3 RESULTS

4.3.1 Accumulation of n-3 fatty acids

The accumulation of EPA in cell phospholipids in HepG2 cells supplemented with EPA was concentration dependent as was that of its elongation product, DPA (Figure 4-1). After supplementation with 10 $\mu\text{g/ml}$ EPA, the level of EPA in cell phospholipids increased 30-fold from $0.30 \pm 0.02\%$ to $9.50 \pm 1.02\%$ total fatty acids. The level of DPA in cell phospholipids increased 12.6-fold from $0.30 \pm 0.02\%$ to $3.79 \pm 0.51\%$ total fatty acids. There was a gradual increase in the level of DHA following supplementation with EPA. Initially, the level of DHA increased with dose, however Figure 4-1 shows that with higher concentrations of EPA (i.e. above 5 $\mu\text{g/ml}$), the accumulation of DHA did not increase. The maximum level of DHA in cell phospholipids was $6.28 \pm 0.91\%$ total fatty acids in HepG2 cells supplemented with 10 $\mu\text{g/ml}$ EPA. These results are summarised in Table 4-1.

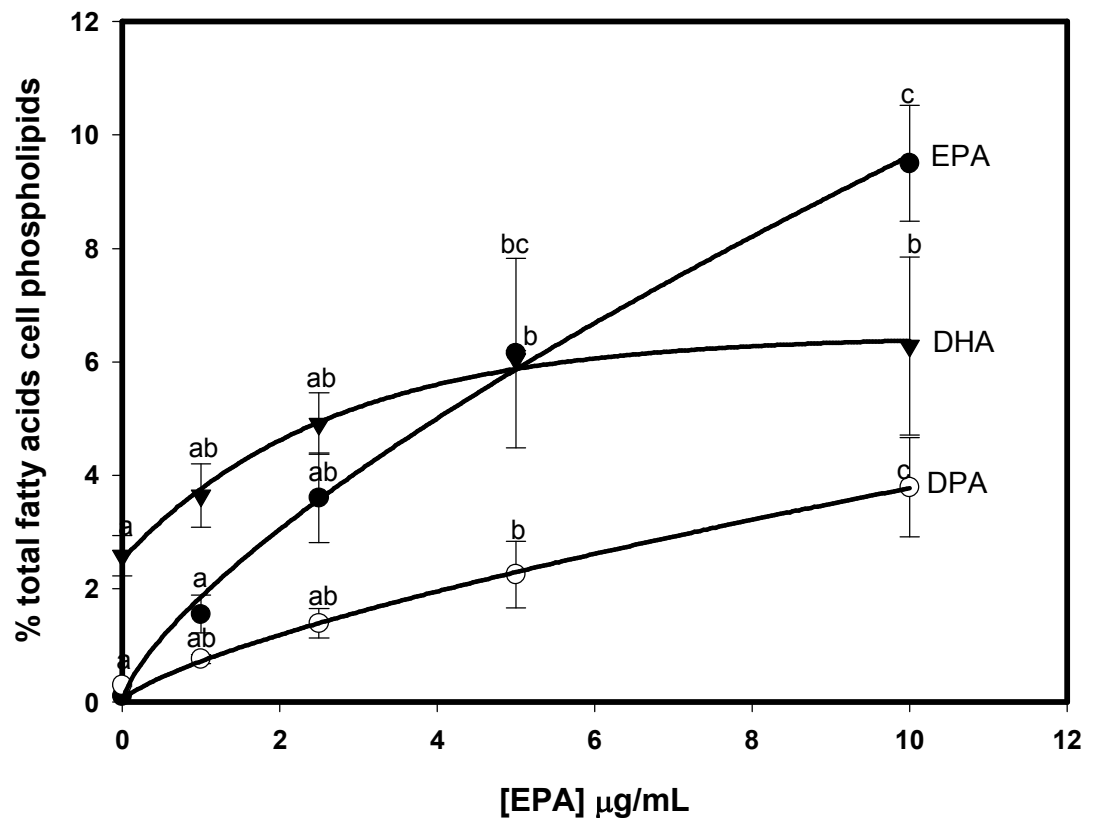


Figure 4-1. The accumulation of eicosapentaenoic acid (20:5n-3, EPA) (●), docosapentaenoic acid (22:5n-3, DPA) (○) and docosahexaenoic acid (22:6n-3, DHA) (▼) in cell phospholipids of HepG2 cells supplemented with EPA.

HepG2 cells were seeded and grown as described. After 72h the medium was replaced with serum-free medium containing increasing amounts of EPA bound to BSA. The cells were harvested for lipid analysis following 48h supplementation with EPA. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group by one-way ANOVA with Bonferroni post-hoc test. Values are means \pm SE of at least three independent experiments with three replicates in each experiment.

Fatty acid	% total fatty acids									
	BSA control		1 µg/ml EPA		2.5 µg/ml EPA		5 µg/ml EPA		10 µg/ml EPA	
Total Saturates	29.75	± 0.60 ^a	31.03	± 0.17 ^{a, b}	32.66	± 0.28 ^{b, c}	34.64	± 0.41 ^c	37.42	± 0.39 ^d
18:1n-9 OA	16.93	± 0.12 ^a	16.17	± 0.60 ^a	15.27	± 0.35 ^{a, b}	13.82	± 0.49 ^b	11.57	± 0.40 ^c
Total Monounsaturates	51.17	± 0.01 ^a	48.64	± 0.55 ^{a, b}	45.10	± 1.40 ^{b, c}	40.66	± 1.33 ^c	34.07	± 1.15 ^d
20:3n-9	5.13	± 0.12 ^a	4.35	± 0.33 ^{a, b}	3.52	± 0.38 ^{b, c}	2.79	± 0.23 ^c	2.22	± 0.18 ^c
Total n-9	29.99	± 0.26 ^a	27.50	± 0.68 ^{a, b}	24.64	± 1.09 ^{b, c}	21.26	± 1.08 ^{c, d}	17.80	± 0.63 ^d
18:2n-6 LA	0.79	± 0.08 ^a	0.80	± 0.097 ^a	0.81	± 0.096 ^a	0.81	± 0.11 ^a	0.81	± 0.15 ^a
18:3n-6	0.10	± 0.02 ^a	0.11	± 0.004 ^a	0.11	± 0.003 ^a	0.10	± 0.003 ^a	0.05	± 0.026 ^a
20:3n-6	0.46	± 0.05 ^a	0.46	± 0.050 ^a	0.46	± 0.042 ^a	0.44	± 0.038 ^a	0.42	± 0.049 ^a
20:4n-6 AA	2.95	± 0.29 ^a	2.87	± 0.24 ^a	2.76	± 0.21 ^a	2.67	± 0.21 ^a	2.61	± 0.32 ^a
22:5n-6 DPA n-6	0.16	± 0.07 ^a	0.20	± 0.0004 ^a	0.17	± 0.012 ^a	0.14	± 0.018 ^a	0.08	± 0.042 ^a
Total n-6	4.53	± 0.46 ^a	4.52	± 0.37 ^a	4.36	± 0.31 ^a	4.21	± 0.31 ^a	4.03	± 0.41 ^a
18:3n-3 ALA	0.01	± 0.005 ^a	0.025	± 0.00 ^a	0.02	± 0.020 ^a	0.025	± 0.00 ^a	0.03	± 0.032 ^a
20:5n-3 EPA	0.10	± 0.003 ^a	1.55	± 0.33 ^a	3.60	± 0.79 ^{a, b}	6.15	± 0.96 ^{b, c}	9.50	± 1.02 ^c
22:5n-3 DPA n-3	0.30	± 0.02 ^a	0.76	± 0.081 ^{a, b}	1.39	± 0.26 ^{a, b}	2.25	± 0.34 ^b	3.79	± 0.51 ^c
22:6n-3 DHA	2.58	± 0.35 ^a	3.64	± 0.56 ^{a, b}	4.91	± 0.54 ^{a, b}	6.04	± 0.090 ^b	6.28	± 0.91 ^b
Total n-3	4.43	± 0.30 ^a	7.25	± 0.85 ^{a, b}	11.04	± 1.44 ^{b, c}	15.34	± 1.14 ^c	20.30	± 0.50 ^d

Table 4-1. Fatty acid composition of HepG2 cell phospholipids of cells supplemented with eicosapentaenoic acid (20:5n-3, EPA).

HepG2 cells were cultured as described and supplemented with increasing concentrations of EPA for 48 h. Values are means ± SE of at least three replicates. Values are means ± SE of at least three independent experiments with three replicates in each experiment. Values with different superscripts are significantly different from each other (p < 0.05) by one-way ANOVA with Bonferroni post-hoc test.

The level of DPA in cells supplemented with DPA also increased linearly with dose, as did the accumulation of its retroconversion product, EPA (Figure 4-2). The level of DPA in cell phospholipids increased from $0.66 \pm 0.34\%$ to $3.51 \pm 0.56\%$ total fatty acids over the concentration range tested (1 – 10 $\mu\text{g/ml}$ DPA). The level of EPA in cell phospholipids increased almost 12-fold from $0.20 \pm 0.13\%$ to $2.36 \pm 0.26\%$ total fatty acids. The accumulation of 24:5n-3 and 24:6n-3 was also measured (Figure 4-3). The accumulation of both 24:5n-3 and 24:6n-3 was dependent on substrate concentration. The level of the D6D substrate, 24:5n-3, increased from $0.15 \pm 0.10\%$ total fatty acids in control cells to $1.33 \pm 0.10\%$ total fatty acids in cells supplemented with 10 $\mu\text{g/ml}$ DPA. The product of $\Delta 6$ desaturation, 24:6n-3, also increased following supplementation with DPA. 24:6n-3 increased 4.4-fold from $0.21 \pm 0.12\%$ total fatty acids in control cells to $0.92 \pm 0.07\%$ total fatty acids in HepG2 cells supplemented with 10 $\mu\text{g/ml}$ DPA. The accumulation of DHA peaked following supplementation with 5 $\mu\text{g/ml}$ DPA and did not increase with higher concentrations of DPA (Figure 4-2), following a similar trend to that observed in cells supplemented with ALA and EPA. At its peak, the level of DHA in cell phospholipids had increased almost 2-fold from $3.36 \pm 0.79\%$ in control cells to $6.53 \pm 0.10\%$ total fatty acids. These results are summarised in Table 4-2.

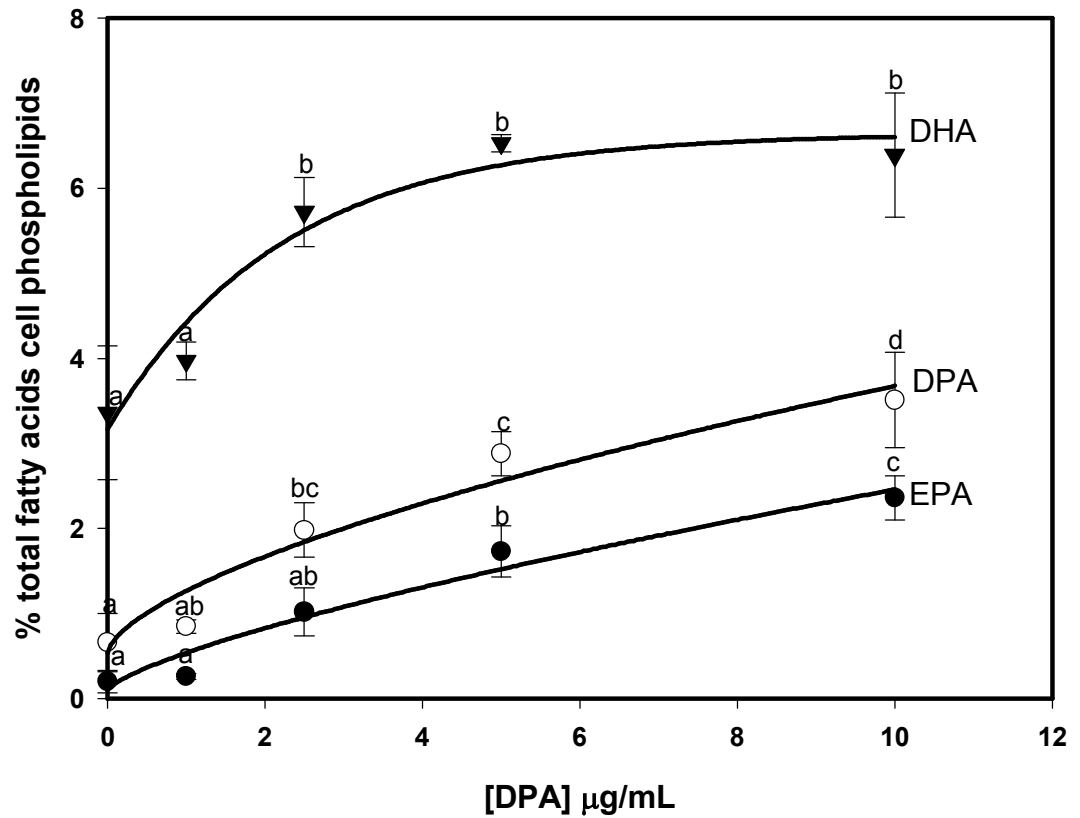


Figure 4-2. The accumulation of docosapentaenoic acid (22:5n-3, DPA) (○), eicosapentaenoic acid (20:5n-3, EPA) (●) and docosahexaenoic acid (22:6n-3, DHA) (▼) in cell phospholipids of HepG2 cells supplemented with DPA.

Conditions as described in Figure 4-1. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group. Values are means \pm SE of at least three independent experiments with three replicates in each experiment.

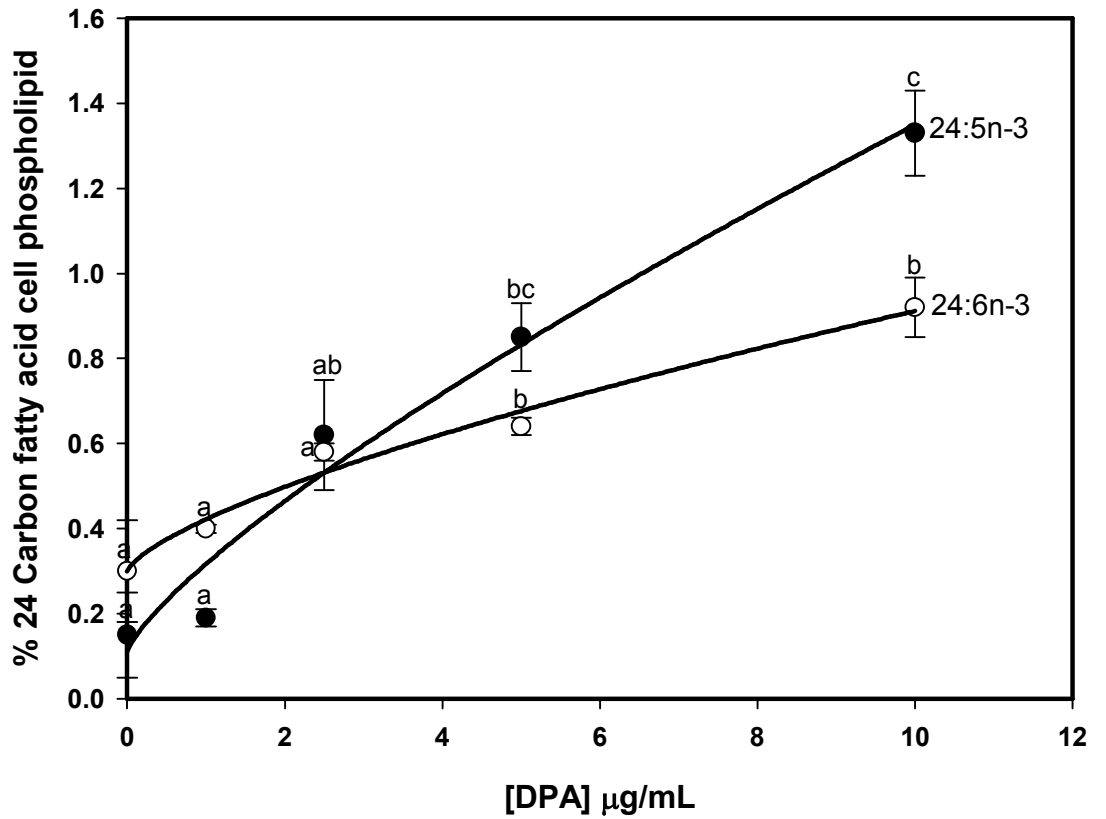


Figure 4-3. The accumulation of 24:5n-3 (●), and 24:6n-3 (○) in cell phospholipids of HepG2 cells supplemented with docosapentaenoic acid (22:5n-3, DPA).

Conditions as described in Figure 4-1. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group. Values are means \pm SE of at least three independent experiments with three replicates in each experiment.

Fatty acid	BSA control	1 µg/ml DPA	2.5 µg/ml DPA	5 µg/ml DPA	10 µg/ml DPA
	% total fatty acids				
Total Saturates	30.34 ± 1.33 ^a	30.65 ± 0.61 ^a	32.55 ± 0.31 ^b	33.76 ± 0.61 ^b	32.39 ± 2.12 ^c
18:1n-9 OA	16.32 ± 0.16 ^a	15.73 ± 0.27 ^a	15.21 ± 0.42 ^{a,b}	14.25 ± 0.38 ^b	11.75 ± 0.60 ^c
Total Monounsaturates	50.47 ± 0.84 ^a	49.99 ± 0.56 ^a	46.15 ± 1.32 ^b	42.89 ± 0.74 ^b	38.33 ± 0.64 ^c
20:3n-9	4.25 ± 0.44 ^a	3.92 ± 0.11 ^b	2.80 ± 0.28 ^c	2.57 ± 0.04 ^{c,d}	2.37 ± 0.12 ^d
Total n-9	27.66 ± 1.81 ^a	26.23 ± 0.43 ^b	23.20 ± 0.43 ^c	21.45 ± 0.17 ^c	19.69 ± 0.85 ^d
18:2n-6 LA	0.79 ± 0.02 ^a	0.78 ± 0.04 ^a	0.89 ± 0.07 ^a	0.89 ± 0.04 ^a	1.00 ± 0.13 ^a
18:3n-6	0.12 ± 0.01 ^a	0.12 ± 0.00 ^a	0.11 ± 0.01 ^a	0.11 ± 0.01 ^a	0.40 ± 0.18 ^a
20:3n-6	0.44 ± 0.01 ^a	0.45 ± 0.02 ^a	0.49 ± 0.04 ^a	0.46 ± 0.02 ^a	0.71 ± 0.17 ^a
20:4n-6 AA	2.82 ± 0.23 ^a	2.84 ± 0.12 ^a	2.86 ± 0.24 ^a	2.77 ± 0.23 ^a	2.52 ± 0.15 ^a
22:5n-6 DPA n-6	0.20 ± 0.02 ^a	0.20 ± 0.01 ^a	0.16 ± 0.002 ^a	0.15 ± 0.003 ^a	0.44 ± 0.18 ^a
Total n-6	4.44 ± 0.30 ^a	4.45 ± 0.18 ^a	4.60 ± 0.37 ^a	4.45 ± 0.31 ^a	6.11 ± 1.32 ^a
18:3n-3 ALA	0.09 ± 0.08	0.01 ± 0.01	0.08 ± 0.04	0.20 ± 0.19	0.34 ± 0.18
20:5n-3 EPA	0.20 ± 0.13 ^a	0.26 ± 0.03 ^a	1.02 ± 0.28 ^{a,b}	1.73 ± 0.30 ^b	2.36 ± 0.26 ^c
22:5n-3 DPA n-3	0.66 ± 0.34 ^a	0.85 ± 0.08 ^{a,b}	1.98 ± 0.32 ^{b,c}	2.88 ± 0.26 ^c	3.51 ± 0.56 ^d
24:5n-3	0.15 ± 0.10 ^a	0.19 ± 0.02 ^a	0.62 ± 0.13 ^{a,b}	0.85 ± 0.08 ^{b,c}	1.33 ± 0.10 ^c
24:6n-3	0.30 ± 0.12 ^a	0.40 ± 0.01 ^a	0.58 ± 0.02 ^a	0.64 ± 0.02 ^b	0.92 ± 0.07 ^b
22:6n-3 DHA	3.36 ± 0.79 ^a	3.97 ± 0.22 ^a	5.72 ± 0.41 ^b	6.53 ± 0.10 ^b	6.39 ± 0.73 ^b
Total n-3	6.19 ± 1.35 ^a	7.14 ± 0.34 ^a	11.15 ± 1.15 ^b	13.86 ± 0.77 ^b	16.63 ± 0.93 ^c

Table 4-2. Fatty acid composition of HepG2 cell phospholipids of cells supplemented with docosapentaenoic acid (22:5n-3, DPA).

HepG2 cells were cultured as described and supplemented with increasing concentrations of DPA for 48 h. Values are means ± SE of at least three independent experiments with three replicates in each experiment. Values with different superscripts are significantly different from each other ($p < 0.05$) by one-way ANOVA with Bonferroni post-hoc test.

4.3.2 Comparison between the accumulation of DHA in HepG2 cells following supplementation with ALA, EPA or DPA

The level of DHA in cell phospholipids was significantly elevated ($p < 0.05$) following supplementation with EPA or DPA compared with cells supplemented with ALA (Figure 4-4). The level of DHA that accumulated in cell phospholipids was directly related to the level of DPA in cell phospholipids with an r^2 coefficient of 0.9801 (Figure 4-5). When DHA was supplemented preformed in the media, the level of DHA in cell phospholipids increased 5.2-fold from $3.14 \pm 0.27\%$ total fatty acids in control cells to $16.26 \pm 0.61\%$ total fatty acids in cells supplemented with $10 \mu\text{g/ml}$ DHA (Figure 4-4).

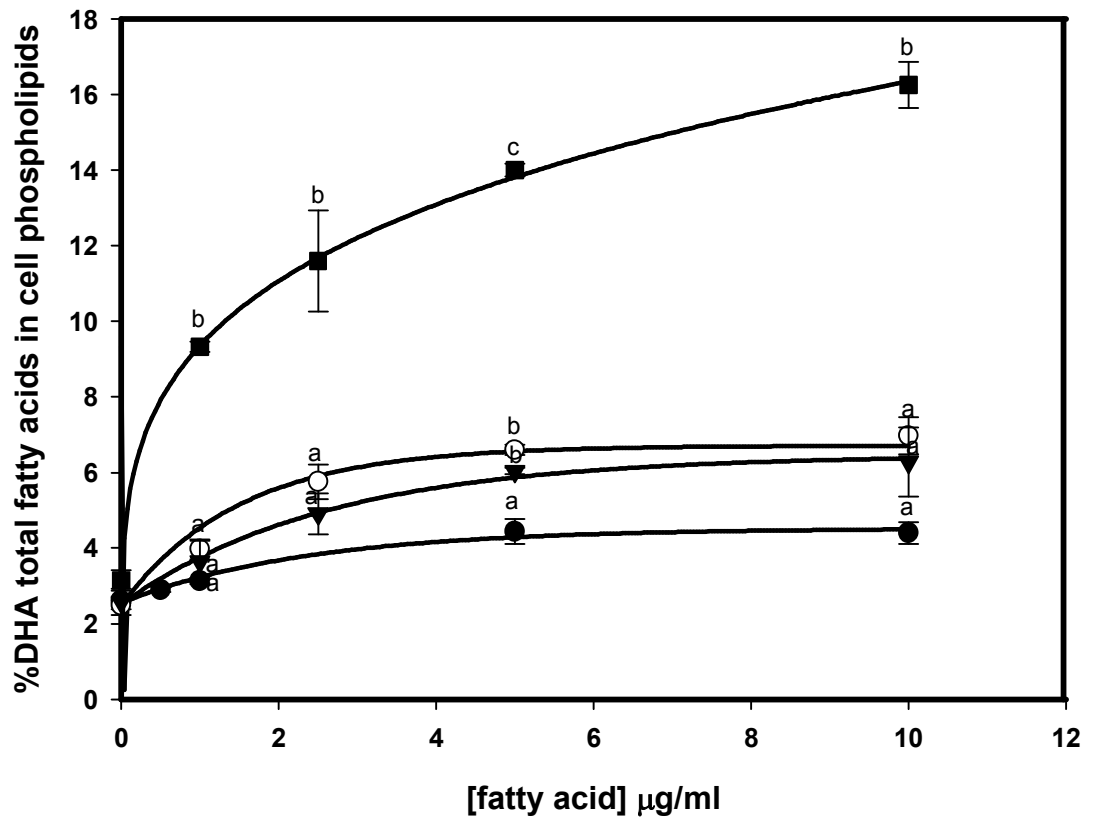


Figure 4-4. The accumulation of docosahexaenoic acid (22:6n-3, DHA) in cell phospholipids of HepG2 cells supplemented with α -linolenic acid (18:3n-3, ALA) (●), eicosapentaenoic acid (20:5n-3, EPA) (▼), docosapentaenoic acid (22:5n-3, DPA) (○) or DHA (■). Conditions as described in Figure 4-1. Values are means \pm SE of at least three independent experiments with three replicates in each experiment. Values with different symbols are significantly different from each other ($p < 0.05$) between each supplementation group. Regression curves have been fitted to the data.

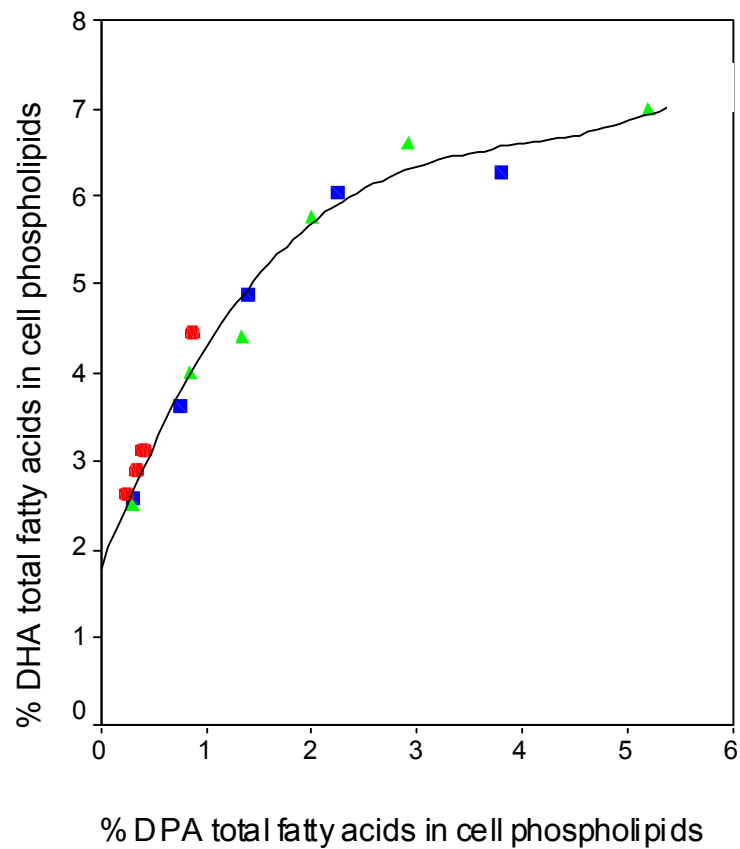


Figure 4-5. The linear relationship between the level of docospentaenoic acid (22:5n-3, DPA) and docosahexaenoic acid (22:6n-3, DHA) in HepG2 cell phospholipids following supplementation with increasing concentrations of α -linolenic acid (18:3n-3, ALA) (■), EPA (■) and DPA (▲). Conditions as described in Figure 4-1. Values are means of at least three independent experiments with three replicates in each experiment. $r^2 = 0.9801$.

4.3.3 Accumulation of n-6 fatty acids

The metabolism and accumulation of the n-6 homologue of DPA, DTA was also examined. The accumulation of DTA in HepG2 cell phospholipids was dose-dependent, increasing from $0.14 \pm 0.03\%$ total fatty acids in control cells to $4.88 \pm 0.54\%$ total fatty acids in cells supplemented with $10 \mu\text{g/ml}$ DTA (Figure 4-6). The retroconversion product of DTA, AA, increased linearly following supplementation with DTA. The level of AA in cell phospholipids increased 2.8-fold, increasing from $2.44 \pm 0.31\%$ total fatty acids in control cells to $6.91 \pm 0.55\%$ total fatty acids in cells supplemented with $10 \mu\text{g/ml}$ DTA. There was a dose-dependent increase in 24:4n-6 and 24:5n-6 in cell phospholipids following supplementation with DTA (Figure 4-7). Similar to the accumulation of DHA in cells supplemented with n-3 fatty acid precursors, the accumulation of the homologous n-6 fatty acid, 22:5n-6, increased following supplementation with increasing concentrations of DTA but further supplementation above $5 \mu\text{g/ml}$ did not result in further increases. These results are summarised in

Table 4-3.

The accumulation of DPA (n-3) and DTA (n-6) in HepG2 cell phospholipids following supplementation with each respective fatty acid paralleled each other (Figure 4-8). The accumulation of the retroconversion products of DTA and DPA, AA and EPA, respectively, paralleled each other, although the level of AA in cell phospholipids was 2.9-fold greater than the accumulation of EPA in DPA-supplemented cells (Figure 4-9).

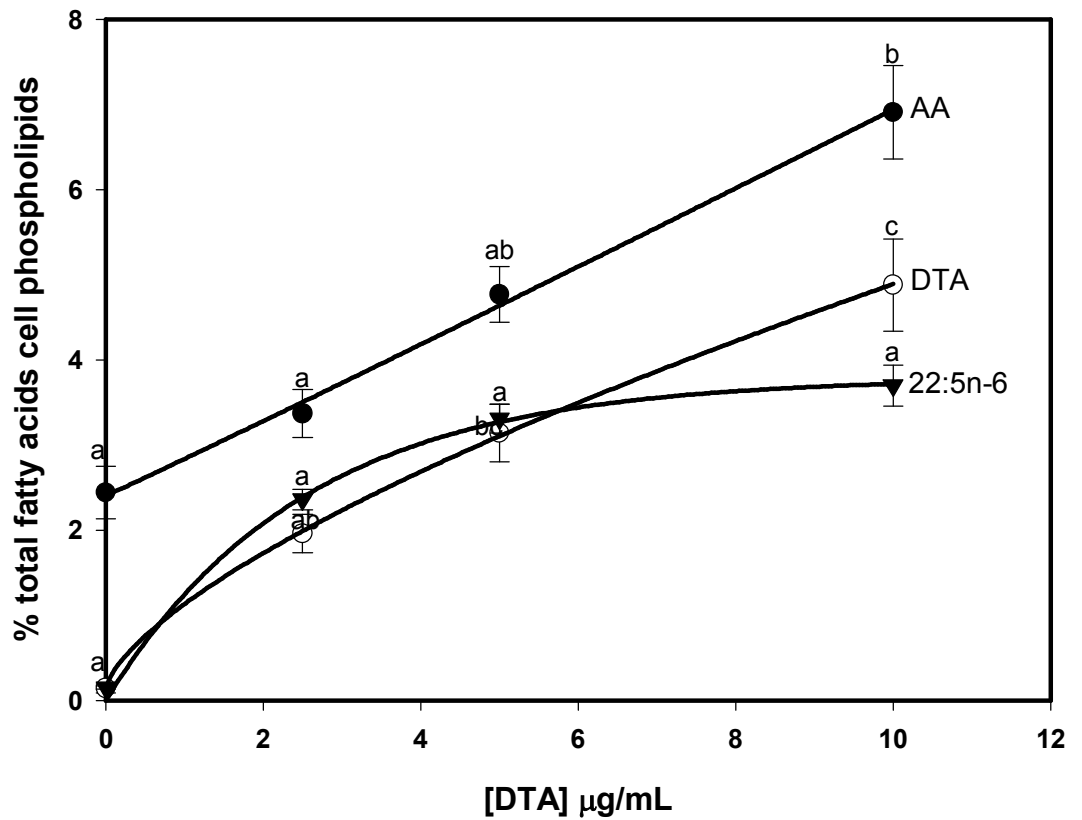


Figure 4-6. The accumulation of arachidonic acid (20:4n-6, AA) (●), docosatetraenoic acid (22:4n-6, DTA) (○) and docosapentaenoic acid (22:5n-6) (▼) in the phospholipid fraction of HepG2 cells supplemented with DTA.

Conditions as described in Figure 4-1. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group. Values are means \pm SE of at least three independent experiments with three replicates in each experiment.

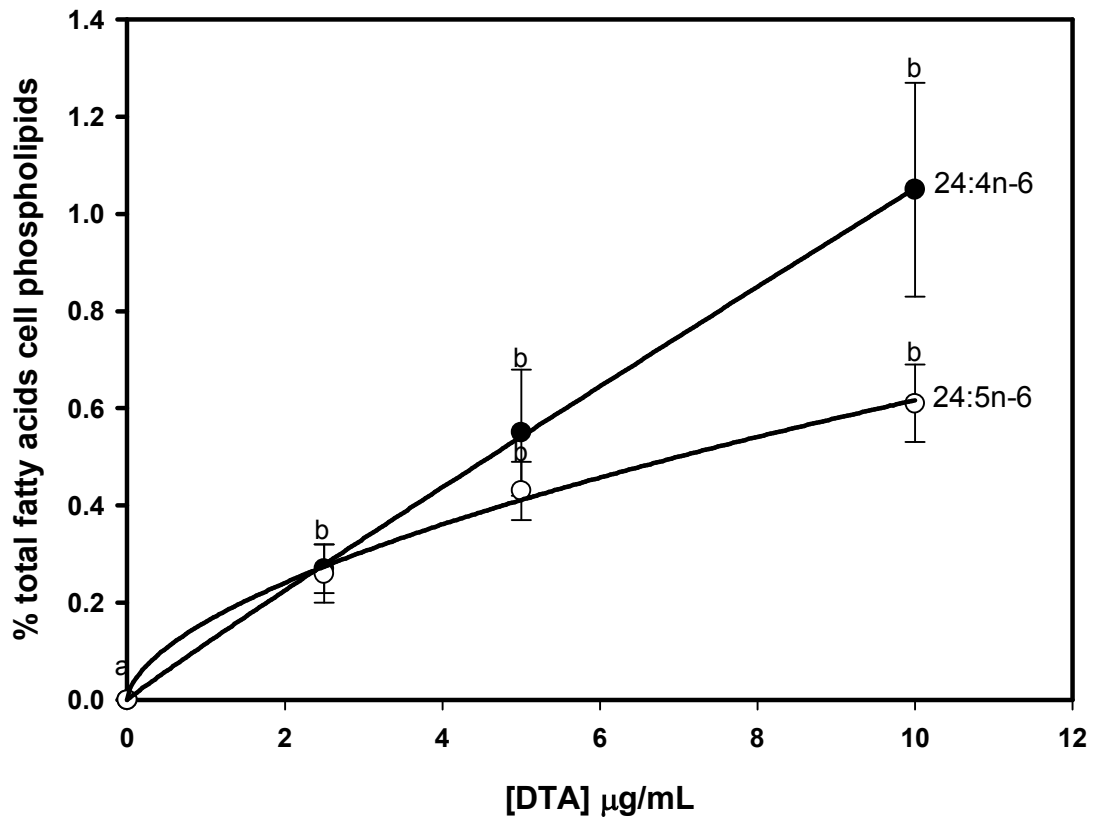


Figure 4-7. The accumulation of 24:4n-6 (●) and 24:5n-6 (○) in the phospholipid fraction of HepG2 cells supplemented with docosatetraenoic acid (22:4n-6, DTA).

HepG2 cells were seeded and grown as described. After 72h the medium was replaced with serum-free medium containing increasing amounts of DTA bound to BSA. The cells were harvested for lipid analysis following 48h supplementation with DTA. Values with different symbols are significantly different from each other ($p < 0.05$) within each fatty acid group. Values are means \pm SE of at least three independent experiments with three replicates in each experiment.

Fatty acid	BSA control	2.5 µg/ml DTA	5 µg/ml DTA	10 µg/ml DTA
Total Saturates	29.40 ± 0.77 ^a	32.70 ± 0.16 ^{a, b}	35.55 ± 0.53 ^b	37.07 ± 1.09 ^b
18:1n-9 OA	17.50 ± 0.34 ^a	15.94 ± 0.13 ^a	15.03 ± 0.40 ^{a, b}	13.34 ± 0.48 ^b
Total Monounsaturates	51.23 ± 0.94 ^a	47.18 ± 0.33 ^{a, b}	43.20 ± 0.44 ^{b, c}	38.86 ± 0.97 ^c
20:3n-9	1.88 ± 1.11 ^a	1.33 ± 0.71 ^a	1.18 ± 0.63 ^a	0.96 ± 0.50 ^a
Total n-9	32.49 ± 1.85 ^a	29.26 ± 1.30 ^a	26.47 ± 1.34 ^a	23.28 ± 1.60 ^a
18:2n-6 LA	0.90 ± 0.05 ^a	0.77 ± 0.07 ^a	0.75 ± 0.07 ^a	0.80 ± 0.11 ^a
18:3n-6	0.06 ± 0.02 ^a	0.07 ± 0.03 ^a	0.08 ± 0.03 ^a	0.08 ± 0.03 ^a
20:3n-6	0.30 ± 0.09 ^a	0.27 ± 0.08 ^a	0.27 ± 0.09 ^a	0.27 ± 0.08 ^a
20:4n-6 AA	2.44 ± 0.31 ^a	3.37 ± 0.28 ^a	4.77 ± 0.33 ^{a, b}	6.91 ± 0.55 ^b
22:4n-6 DTA	0.20 ± 0.09 ^a	1.96 ± 0.23 ^{a, b}	3.14 ± 0.34 ^{b, c}	4.88 ± 0.54 ^c
22:5n-6 DPAn-6	0.20 ± 0.09 ^a	0.17 ± 0.09 ^a	0.16 ± 0.08 ^a	0.14 ± 0.08 ^a
24:4n-6	0.025 ± 0.00 ^a	0.27 ± 0.05 ^b	0.55 ± 0.13 ^b	1.05 ± 0.22 ^b
24:5n-6	0.025 ± 0.00 ^a	0.26 ± 0.06 ^b	0.43 ± 0.06 ^b	0.61 ± 0.08 ^b
Total n-6	7.93 ± 1.09 ^a	10.38 ± 0.83 ^{a, b}	12.84 ± 1.13 ^{a, b}	17.14 ± 1.29 ^b
18:3n-3 ALA	0.11 ± 0.04 ^a	0.11 ± 0.04 ^a	0.12 ± 0.04 ^a	0.13 ± 0.04 ^a
20:5n-3 EPA	0.12 ± 0.02 ^a	0.11 ± 0.01 ^a	0.09 ± 0.01 ^a	0.08 ± 0.01 ^a
22:5n-3 DPAn-3	0.27 ± 0.08 ^a	0.25 ± 0.07 ^a	0.22 ± 0.06 ^a	0.19 ± 0.05 ^a
24:5n-3	1.98 ± 0.37 ^a	1.63 ± 0.21 ^a	1.49 ± 0.17 ^a	1.26 ± 0.15 ^a
24:6n-3	0.04 ± 0.01 ^a	0.04 ± 0.02 ^a	0.03 ± 0.01 ^a	0.02 ± 0.01 ^a
22:6n-3 DHA	0.23 ± 0.03 ^a	0.18 ± 0.02 ^a	0.14 ± 0.02 ^a	0.10 ± 0.01 ^a
Total n-3	3.44 ± 0.71 ^a	2.90 ± 0.42 ^a	2.68 ± 0.35 ^a	2.22 ± 0.27 ^a

Table 4-3. Fatty acid composition of HepG2 cell phospholipids of cells supplemented with docosatetraenoic acid (22:4n-6, DTA).

HepG2 cells were cultured as described and supplemented with increasing concentrations of DTA for 48 h. Values are means ± SE of at least three independent experiments with three replicates in each experiment. Values with different superscripts are significantly different from each other ($p < 0.05$) by one-way ANOVA with Bonferroni post-hoc test.

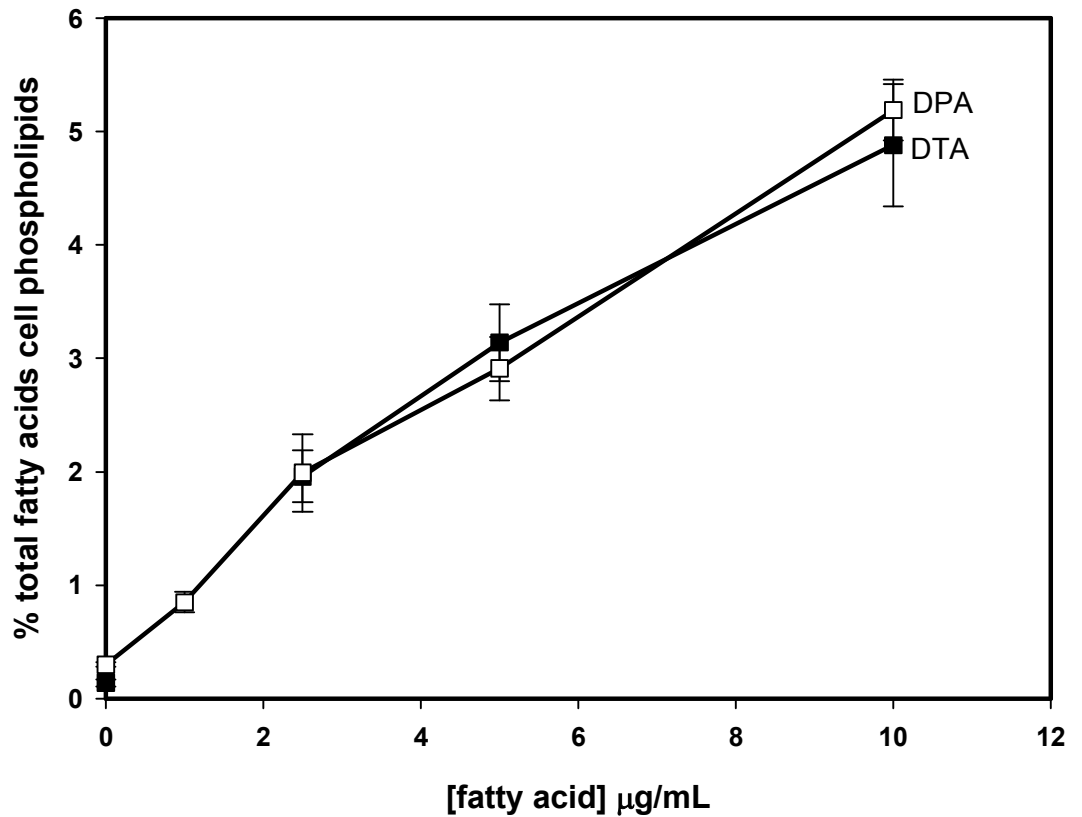


Figure 4-8. The accumulation of docosatetraenoic acid (22:4n-6, DTA) (■) and docosapentaenoic acid (22:5n-3, DPA) (□) in the phospholipid fraction of HepG2 cells supplemented with DTA or DPA, respectively.

HepG2 cells were seeded and grown as described. After 72h the medium was replaced with serum-free medium containing increasing amounts of DTA bound to BSA. The cells were harvested for lipid analysis following 48h supplementation with DTA. Values are means \pm SE of at least three independent experiments with three replicates in each experiment.

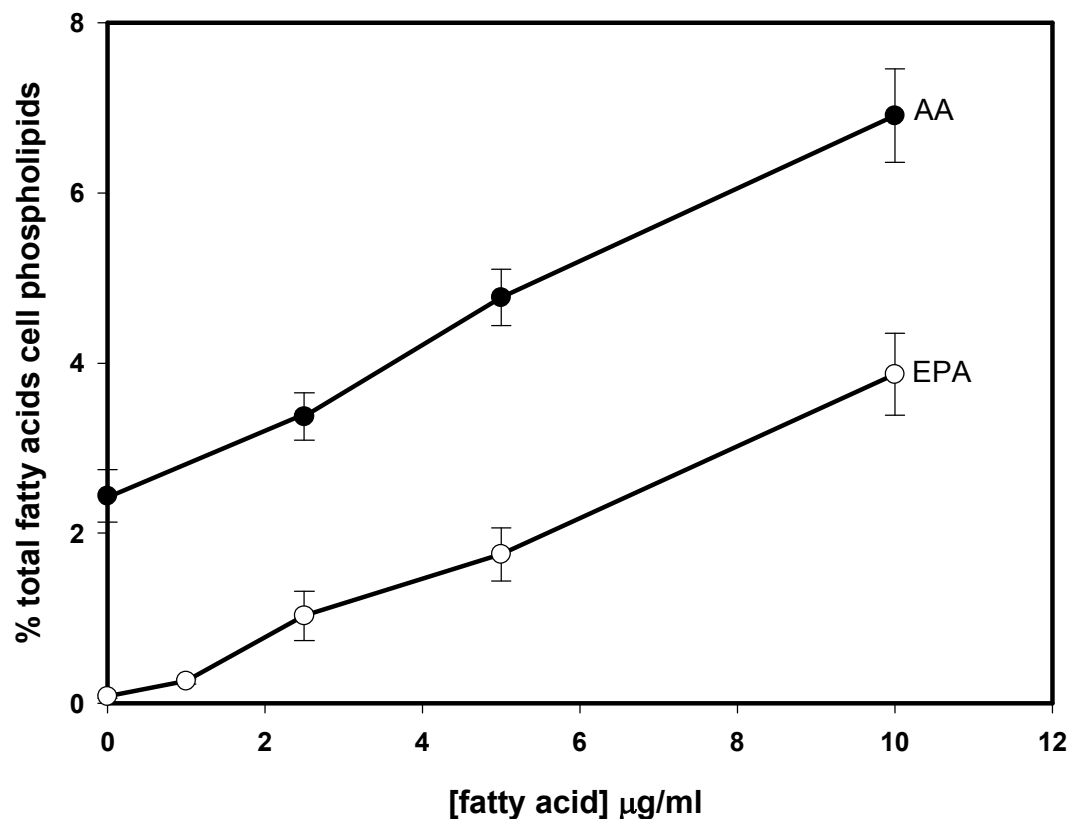


Figure 4-9. The accumulation of eicosapentaenoic acid (20:5n-3, EPA) (○) and arachidonic acid (20:4n-6, AA) (●) in the phospholipid fraction of HepG2 cells supplemented with docosapentaenoic acid (22:5n-3, DPA) or docosatetraenoic acid (22:4n-6, DTA), respectively.

HepG2 cells were seeded and grown as described. After 72h the medium was replaced with serum free medium containing increasing amounts of DPA or DTA bound to BSA. The cells were harvested for lipid analysis following 48h supplementation with each respective fatty acid. Values are means \pm SE of at least three independent experiments with three replicates in each experiment.

4.3.4 Competition between fatty acids for incorporation into membranes

The fatty acid composition of HepG2 cells co-supplemented with DHA and increasing concentrations of ALA, LA or EPA was measured to examine the competition between fatty acids on the accumulation of DHA into cell lipids. The incubation time in these experiments was reduced to 2 h to limit the conversion of the supplemented fatty acid. After 2 h supplementation with 5 µg/ml DHA, the level of DHA in HepG2 cell phospholipids was $8.09 \pm 0.44\%$ total fatty acids. The level of DHA in cell phospholipids was unaffected by the addition of increasing concentrations of LA or EPA ($p > 0.05$) (Figure 4-10).

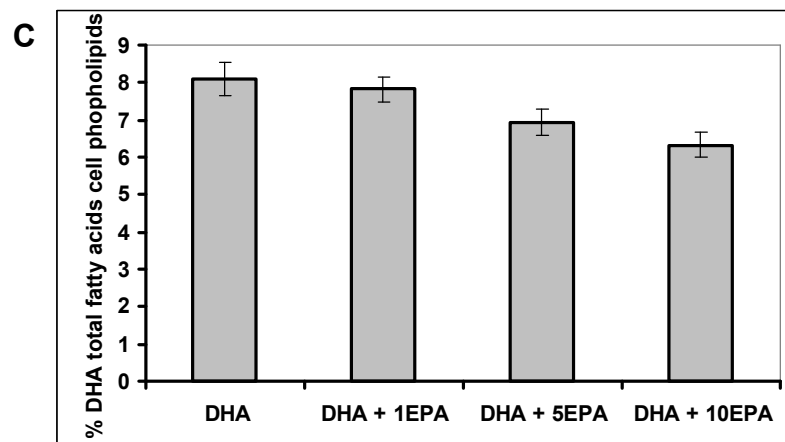
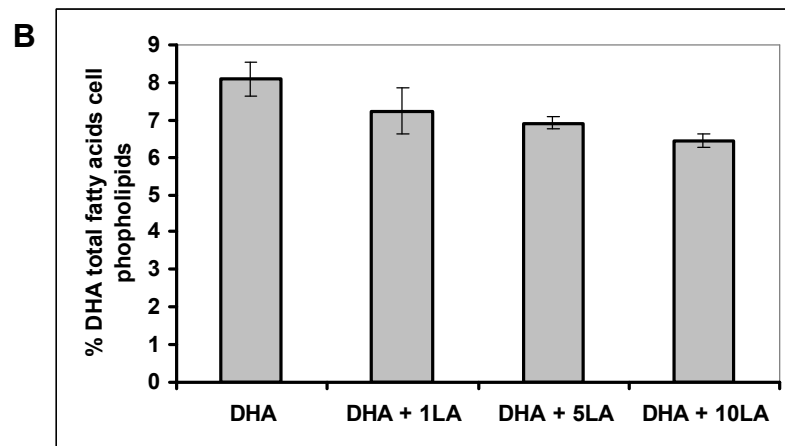
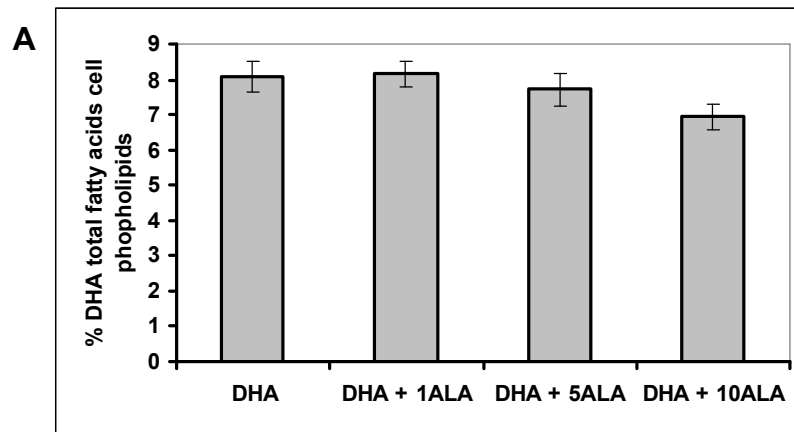


Figure 4-10. The accumulation of docosahexaenoic acid (22:6n-3, DHA) in phospholipids of HepG2 cells supplemented with DHA alone or in addition to increasing concentrations of α -linolenic acid (18:3n-3, ALA), linoleic acid (18:2n-6, LA) or eicosapentaenoic acid (20:5n-3, EPA).

HepG2 cells were seeded and grown as described. After 72 h the medium was replaced with serum-free medium containing 5 $\mu\text{g/ml}$ DHA, bound to BSA, alone or with the addition of either 1, 5 or 10 $\mu\text{g/ml}$ ALA (A), LA (B) or EPA (C). The cells were harvested for lipid analysis following 2 h supplementation with the fatty acid/s. Values are means \pm SE of at least three independent experiments with three replicates in each experiment. Values are not statistically different from each other ($p > 0.05$) by one-way ANOVA with Bonferroni post-hoc analysis.

4.4 DISCUSSION

In this chapter, EPA and DPA were identified as more efficient n-3 fatty acid substrates in elevating the content of DHA in cell phospholipids compared with ALA. The absence of competition between ALA and 24:5n-3 for D6D in EPA- and DPA-supplemented cells may have contributed to the elevated accumulation of DHA. A strong predictor for the accumulation of DHA in cell phospholipids from n-3 fatty acid precursors was the level of DPA in cell phospholipids (Figure 4-5). DPA is the most immediate precursor to DHA and readily accumulates in cell phospholipids following supplementation with EPA or DPA. The elevated accumulation of DHA from EPA or DPA compared with ALA has also been demonstrated in the human neuroblastoma cell line, SH-SY5Y (181), cerebral endothelial cells (182;183) and retinal endothelial cells (183). The level of DHA in brain and retina is reportedly several hundred-fold more than the level of EPA (179), therefore studies in neural tissue may not reflect the conversion of fatty acids in HepG2 cells. Given the potential for tissue specific accumulation of individual fatty acids, particularly retinal and neural cells (129;184;185), a thorough examination of the accumulation of DHA from EPA and DPA in HepG2 cells is warranted.

EPA was converted to DHA more so than ALA in HepG2 cells, however, in humans, supplementation with pure EPA does not affect the level of DHA in tissues suggesting a greater level of regulation *in vivo* (186-188). James *et al.* (2003) (153) compared the fatty acid composition of plasma phospholipids between subjects supplemented with stearidonic acid (SDA, 18:4n-3) or EPA. Dietary supplementation with SDA or EPA significantly increased the level of EPA and DPA in erythrocyte and plasma phospholipids, however, the concentration of DHA was unaffected (153). Other studies show that supplementation with approximately 4 g/d of pure EPA ethyl ester results in significant increases in EPA concentration in plasma lipids, but no increase in the concentration of DHA (186-189). The potential role of DPA in elevating n-3 LCPUFA status has been largely ignored despite recent research which shows that DPA contributes almost 30% total n-3 LCPUFA in our diet (2). The results presented in this Chapter indicates a need to assess the efficacy of DPA in elevating the concentration n-3 LCPUFA, particularly DHA, in humans.

Irrespective of the substrate fatty acid, the accumulation of DHA from ALA, EPA or DPA reached a plateau with increasing concentration of the substrate. The incorporation of EPA into membrane lipids may divert its continued elongation and progression of EPA towards the synthesis of DHA. In ALA-, EPA- and DPA-supplemented HepG2 cells, the level of EPA in cell phospholipids increased 47-, 30- and 12-fold, respectively, and represented the greatest increase in the level of PUFA in cell phospholipids compared to other PUFA in cell phospholipids.

It has been shown that the primary product of DPA metabolism in retinal and cerebral endothelial cells is EPA, followed by DHA, 24:5n-3 and 24:6n-3 (183). The entry of DPA into peroxisomal β -oxidation and subsequent retroconversion to EPA

may also divert the conversion of DPA away from the synthesis of DHA to contribute to the limited accumulation of DHA in cell phospholipids. In cells supplemented with DTA, the preferential incorporation of 20:4n-6 may limit the conversion of DTA to 22:5n-6 and the subsequent incorporation of 22:5n-6 into cell lipids, analogous to the way EPA is preferentially incorporated into cell lipids, thus preventing its continued synthesis towards DHA in cells supplemented with DPA. The preferential esterification of [1-¹⁴C] 20:4n-6 from [3-¹⁴C] 22:4n-6 (DTA) rather than continued β -oxidation has been demonstrated using microsomes and 1-acyl-GPC (190;191). The preferential incorporation of n-3 and n-6 20 carbon fatty acids into cell phospholipids may be important determinants of the fatty acid composition of cell membranes and the conversion of substrate fatty acids to n-3 and n-6 LCPUFA.

Regardless of substrate, the accumulation of DHA in cells supplemented with n-3 fatty acid, and its homologue, 22:5n-6, in cells supplemented with DTA, approached a plateau. The linear accumulation of the elongation products of EPA and DPA, including DPA and 24:5n-3, respectively, suggests that the elongation of EPA and DPA was not limited (Figure 4-1, Figure 4-3). The accumulation of the D6D product, 24:6n-3, in DPA- supplemented cells was also linear suggesting that the activity of D6D was not limited at the concentrations used here (Figure 4-3). Similarly, the accumulation of 24:4n-6 and 24:5n-6 was linear (Figure 4-7). It may be inferred, therefore, that the level of DHA in cell phospholipids may be regulated by events post-D6D, including translocation of 24:6n-3 to the peroxisome (96), β -oxidation of 24:6n-3 to DHA, or translocation of DHA out of the peroxisome for incorporation into cellular lipids.

The incorporation of preformed fatty acids into diacylglycerol for use in phospholipid synthesis *de novo* may also influence the fatty acid composition of cell phospholipids (192). The fatty acyl composition of the diacylglycerol molecule utilised in the CDP-choline pathway determines the fatty acyl array for phosphatidylcholine synthesis (127). The acyl chain composition of phospholipids may also be remodelled by a deacylation-reacylation cycle (193). In this way, the fatty acid composition of cell phospholipids may be modified to maintain the typical fatty acid composition of the tissue (193). In the heart, a large proportion of phosphatidylcholine undergoes remodelling in order to acquire the proper acyl groups to maintain electrical signal generation and conduction imperative to cardiac function (194). The remodelling of acyl chains may explain, in part, the limited effect of fatty acid co-supplementation on the fatty acid composition of HepG2 cell phospholipids (Figure 4-10). The marked increase in the accumulation of DHA in cells supplemented with preformed DHA and the marginal reduction in the level of DHA following co-supplementation with other fatty acids suggests that the supply of diacylglycerol for use in the CDP-choline pathway was not limiting under the conditions used here.

In conclusion, this chapter described the accumulation of n-3 and n-6 LCPUFA in HepG2 cell phospholipids. The synthesis of 22:5n-6 from 24:4n-6 proceeds via a pathway analogous to the synthesis of DHA from DPA (195). The similar rates of accumulation of DTA and DPA, and the paralleled accumulation of their respective products of retroconversion (AA and EPA) supports the proposed common use of enzymes involved in fatty acid conversion by n-3 and n-6 fatty acids. The accumulation of n-6 fatty acids in HepG2 cell phospholipids showed marked congruency with the accumulation of n-3 fatty acids (Figure 4-8, Figure 4-9). It was

hypothesised that HepG2 cells supplemented with n-3 fatty acid substrates downstream of ALA would accumulate DHA more readily as the competition between ALA and 24:5n-3 for D6D would be bypassed. HepG2 cells supplemented with EPA or DPA did indeed show significantly higher levels of DHA in phospholipids, however, the accumulation of DHA was limited with increasing concentrations of substrate. Regulation of the expression and activity of D6D may partly explain the non-linearity in the accumulation of DHA from n-3 LCPUFA precursors and is addressed in the following chapter. The linear accumulation of EPA and AA in cells supplemented with EPA, DPA and DTA, respectively, suggests that the preferential incorporation may divert the conversion of these fatty acids to LCPUFA. This will be addressed in Chapter 6.

5 D6D mRNA abundance and DHA incorporation in HepG2 cells

5.1 INTRODUCTION

The synthesis of LCPUFA from ALA and LA has been well documented, yet regulation of the pathway, particularly the regulation of the conversion of ALA to DHA, remains a focus of investigation. There are several plausible explanations for the non-linearity between the supplementation of n-3 fatty acid substrates and their conversion to DHA *in vitro*. Fatty acids may enter several pathways including direct incorporation into structural lipids, β -oxidation and conversion to LCPUFA (151). D6D is an integral enzyme in the conversion of 18 carbon fatty acids to LCPUFA (87;94) and is subject to regulation at the transcriptional level (107). Dietary studies in animals indicate that hepatic D6D mRNA expression and activity is induced by diets low in essential fatty acids and suppressed by diets rich in polyunsaturated vegetable or marine oils (80;105;107;108). Changes in the level of D6D mRNA may affect the amount of D6D protein available for the synthesis of DHA from n-3 fatty acid substrates. However, it is unclear from the literature the extent that the down-regulation of D6D gene expression affects the level of LCPUFA in cell membranes. This chapter aimed to examine the effect of fatty acid supplementation on the mRNA abundance and protein expression of D6D and the fatty acid composition of HepG2 cell phospholipids.

5.2 MATERIALS AND METHODS

The materials and methods used were those described in Chapter 2, with the following additional methods.

5.2.1 Cell culture

HepG2 cells were cultured and seeded into six-well plates as described in Section 3.2.1. After 72 h, the medium was removed and replaced with serum-free DMEM supplemented with 20 µg/ml OA, LA, ALA, AA or EPA bound to essentially fatty acid-free BSA. Stock solutions of these fatty acids were prepared in ethanol at a concentration of 10 mg/ml and diluted in serum-free medium to achieve the final desired concentration. After 48 h incubation with the respective fatty acids, cells were harvested for lipid (described in Section 2.2.7), RNA (described in Section 5.2.3) or protein extraction (described in Section 5.2.5). The mRNA expression of D6D was examined after 1, 2, 6, 12, 24 and 48 h supplementation with 20 µg/ml ALA.

5.2.2 Fatty acid analysis

Lipids were extracted from cells following procedures described in Chapter 2.

5.2.3 RNA extraction

Total RNA was isolated using a commercially available kit (Qiagen, CA, USA). To remove contaminating genomic DNA, RNA samples were treated with RNase-Free DNase (Qiagen, CA, USA). The yield of purified RNA samples was determined by absorbance at 260 nm. Equal loading and integrity of RNA samples were confirmed by ethidium bromide fluorescence of ribosomal 18S and 28S RNA.

5.2.4 Real-time RT-PCR

Quantitative real-time PCR analysis (Corbett Rotorgene 3000, Sydney, Australia) was performed using SYBR® green fluorescence was used to determine the relative abundance of D6D mRNA. Two µg of total RNA was reverse transcribed with random hexamers according to the manufacturer's instructions (Ambion, Austin, TX,

USA). Each amplification mixture (20 μ l) contained 0.5 μ l of cDNA, 90 nM forward D6D primer (5'-CTGCCAACTGGTGGGAATCATC), 90 nM reverse D6D primer (5'-ACAAACACGTGCAGCATGTTC) and PCR master mix (Stratagene, La Jolla, CA, USA). Published primer sequences were used for the forward and reverse D6D primers (164). The PCR product was estimated at 93 bp and was confirmed on a 2% agarose gel in comparison to the DNA marker, pUC 19, (AMS Biotechnology, Oxon, UK). The mRNA abundance relative to that of the house keeping gene, cyclophilin A, was measured using the comparative C_T (Threshold cycle) method. The forward and reverse cyclophilin A primers were 5'-GGTTGGATGGCAAGCATGTG and 5'-TGCTGGTCTTGCCATTCCTG, respectively. Cyclophilin A, a ubiquitously expressed gene, regulates protein folding and is routinely used as a housekeeping gene (196;197). Thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing of primers at 55°C for 30 s and extension at 72°C for 30 s. After 35 cycles, a melt curve was generated by heating the sample to 72°C for 5 s and 99°C for 5 s.

Six replicates of cDNA amplification were performed. The PCR amplification products were sent to the Flinders University DNA Sequencing Facility, Adelaide, Australia, for direct sequencing to confirm their identities with the D6D and cyclophilin A gene sequences, respectively. Sequence analysis was performed using GeneDoc Multiple Sequence Alignment Editor Version 2.6 Software (Boston, MA, USA). The D6D reverse and forward sequences were aligned with the reported D6D gene sequence (198) (GenBank Accession Number AF084559).

5.2.5 Protein extraction

At the end of supplementation with fatty acids, the medium was aspirated and the cells were washed twice with ice-cold PBS. Cells were collected in ice-cold Radio Immuno Precipitation Assay (RIPA) buffer (150 mM NaCl, 64 mM Tris base, 1% NP-40 and 0.5% sodium deoxycholate) and centrifuged at 14,000 g for 15 min in a pre-cooled ultracentrifuge. Total protein of each cell lysate was determined by the bicinchoninic acid (BCA) colorimetric method (Pierce, Rockford, IL, USA) (199;200) with BSA as the standard.

5.2.6 SDS-PAGE

50 µg protein was reduced in loading buffer containing 5% (v/v) β-mercaptoethanol and the samples were denatured at 95°C for 1 min. Reduced protein samples were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide resolving gel and a 4% acrylamide stacking gel under constant voltage (100 V) for 1 h. The proteins were identified by comparison of the apparent molecular weights with those of a protein ladder (Invitrogen, Carlsbad, CA) that were electrophoresed under identical conditions.

5.2.7 Western blot analysis

Fractionated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Whatman Group, Dassel, Germany). The membrane was probed with two polyclonal anti rat D6D sera, both of which were kindly donated by Professor Philippe Legrand (Laboratoire de Biochimie, INRA-ENSA, 35590 Saint-Gilles, France) (95). To determine an appropriate dilution of primary antibody in applications with the HepG2 cell line, 50 µg of reduced protein was fractionated by SDS-PAGE and electrophoretically transferred on to nitrocellulose membranes. The membrane was saturated in PBS containing 0.1%

Tween-20 (Sigma, St Louis, MO, USA) and 5% skim milk powder (w/v) for 1 h at room temperature. The membrane was then probed with serial dilutions of serum 1, serum 2, or both sera, ranging from 1:16000 to 1:1000, diluted in PBS containing 0.1% Tween-20 and 5% skim milk powder (w/v) at 4°C over night. Rabbit antibodies were revealed with horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulins (Dako Corp., Glostrup, Denmark) diluted in PBS containing 0.1% Tween-20 and 5% skim milk powder (w/v) at 1:2000 for 1 h at room temperature. Washes were performed in PBS containing 0.1% Tween-20. Peroxidase activity was revealed by following the procedure provided for the ECL Super Signal West Pico detection kit (Pierce, Rockford, IL, USA). High background fluorescence was observed when the membrane was probed with a 1:2000 dilution of the two anti-rat D6D primary antibodies (serum 1 and 2) and a 1:2000 dilution of the secondary antibody. To determine whether this fluorescence was due to non-specific binding of the primary and secondary antibodies, the membrane was probed with the secondary antibody only. Figure 5-1 (A) indicates that the high background fluorescence was from the primary antibodies. To reduce the high background fluorescence, an optimal dilution of the primary antibodies was determined by probing the membrane with serial dilutions of serum 1, serum 2 or both sera. Figure 5-1 (B) indicates that optimal sensitivity, which was defined by minimal background fluorescence and the identification of bands, was achieved with a 1:1 mixture of serum 1 and 2 diluted to 1:1000. The integrity of extracted protein was assessed by probing the membrane with a β -tubulin primary antibody, which was detected with the horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulins, as described. Figure 5-2 indicates that the protein extracted from HepG2 cells was not degraded, as a single β -tubulin band of the expected size (55 kDa) was detected when the membrane was probed with anti- β -tubulin antibodies. The use of these anti-rat D6D antibodies in

COS-7 cells transfected with rat D6D previously revealed a single band at 45 kDa (95).

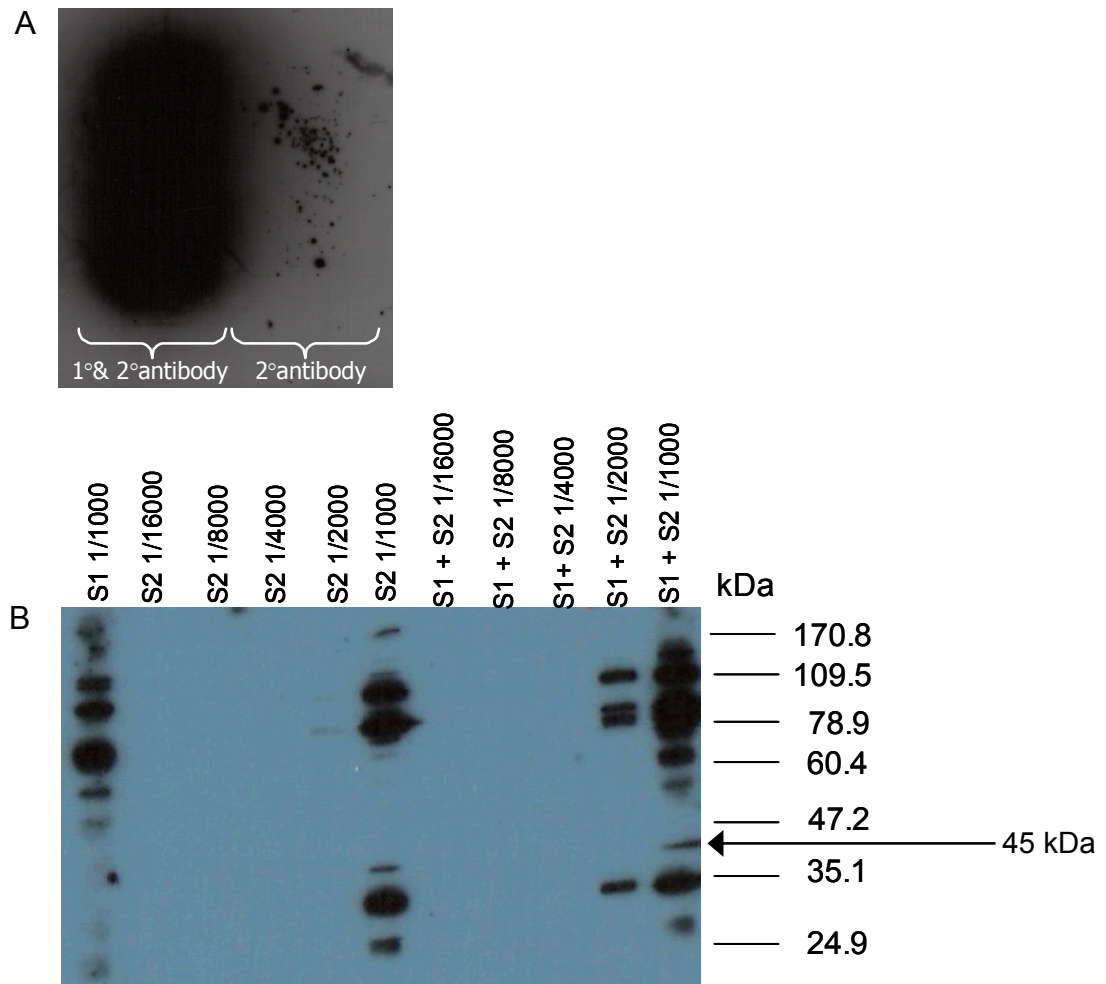


Figure 5-1. Western blot analysis of $\Delta 6$ desaturase in HepG2 cell protein lysates. Total cell protein lysates (50 μ g) from HepG2 cells were resolved by SDS-PAGE and blotted. (A) The blot was probed with anti-rat $\Delta 6$ desaturase sera (serum 1 (S1) and serum 2(S2)) at 1:2000 dilution (1° antibody) and revealed with horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulins (2° antibody) diluted to 1:2000. Exposure time was 30 s. (B) The blot was probed with serial dilutions of anti-rat $\Delta 6$ desaturase sera (S1 and S2), as indicated, and detected with horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulins diluted to 1:2000. The previous use of these anti-rat D6D antibodies in COS-7 cells

transfected with rat $\Delta 6$ desaturase revealed a single band at 45 kDa (95). Exposure time was 1 min.

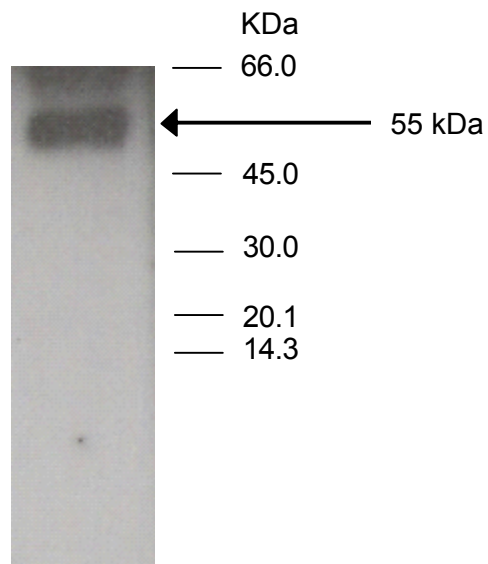


Figure 5-2. Detection of β -tubulin in HepG2 cells. Total cell protein lysates (50 μ g) were resolved by SDS-PAGE and blotted. The blot was probed with anti- β -tubulin antibody at 1:1000 dilution. The expected size of β -tubulin is 55 kDa. Detection was performed using chemifluorescence after 5 s.

5.2.8 Statistical analysis

Fatty acid analyses were performed on means of at least three independent experiments. The mean D6D mRNA abundance was compared from at least five separate replicates. ANOVA and Bonferroni post hoc statistical tests (SPSS Inc Chicago, IL, USA) were used to compare means following fatty acid supplementation. Statistical significance was defined as $p < 0.05$. Data are expressed as mean \pm SE.

5.3 RESULTS

5.3.1 Real-time RT PCR analysis

5.3.1.1 RNA extraction

The yield, purity and quality of extracted RNA were determined by ethidium bromide fluorescence before use in downstream applications (Figure 5-3).

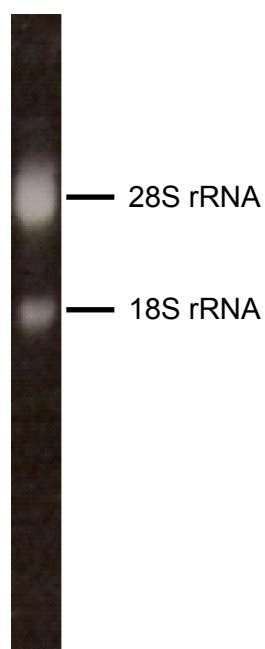


Figure 5-3. Purity and integrity of RNA extracted from HepG2 cells.

RNA was extracted from HepG2 cells using a commercial kit (Qiagen, CA, USA) according to the manufacturer's instructions. The purity and integrity of the extracted RNA was examined by electrophoresis on a formaldehyde-agarose gel, followed by staining with ethidium bromide.

5.3.1.2 Primer efficiency

The amplification efficiency of each primer pair (D6D and cyclophilin A) was compared by measuring the change in C_T with template dilution. Curves were generated from cDNAs made from increasing amounts of total RNA (0.125, 0.25, 0.5, 1, 2 μg). The change in threshold cycle ($\Delta C_T = C_T \text{ D6D} - C_T \text{ cyclophilin A}$) was calculated for each dilution (*Y-axis*) and graphed against the logarithm of template concentration (*X-axis*) (Figure 5-4). The amplification efficiency for each gene was calculated according to the following equation: $E = 10^{(-1/S)}$, where S is the slope of

the curve (201). The slope of the line was < 0.1 , therefore the amplification efficiencies of each gene were equal and the ΔC_T method was used to analyse the data (Figure 5-4).

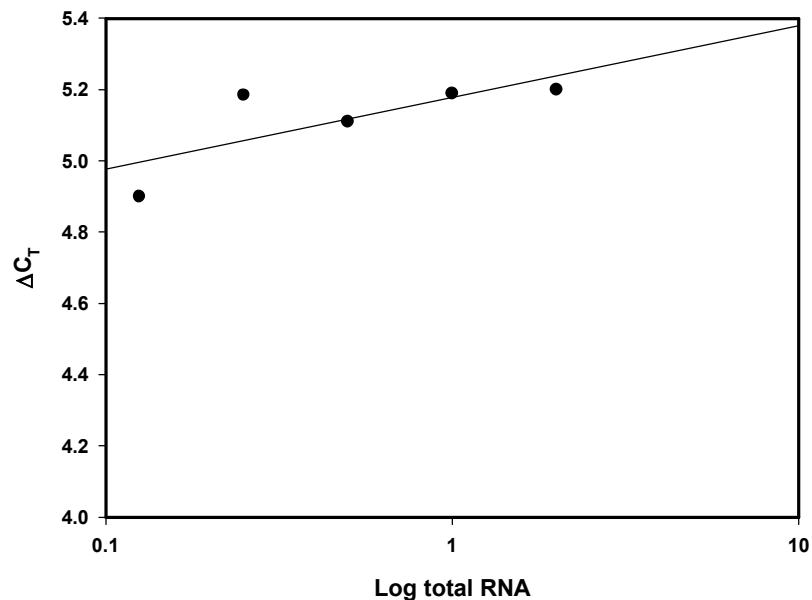


Figure 5-4. PCR amplification efficiency of $\Delta 6$ desaturase and cyclophilin A primers. The difference in C_T values (ΔC_T) was plotted against log template amount. The difference in PCR efficiency was determined by calculating the slope of the line (0.087).

5.3.1.3 PCR Product

The D6D PCR product was analysed by Agarose gel electrophoresis to examine the presence of any contaminating genomic DNA in the isolated RNA or the formation of primer-dimers. Melt curves were also performed at the end of 35 cycles to check for these contaminants. Agarose gel electrophoresis (Figure 5-5 A) and melt curve analysis (Figure 5-6 C) of the D6D PCR product indicated that the primers were specific for D6D and there was no contaminating genomic DNA as the primers did not form primer-dimers. The D6D PCR product was detected at its predicted size of

93 bp. The sequence of the D6D forward and reverse products is shown in Figure 5-5 (B) and agrees with the reported cDNA sequence (GenBank Accession Number AF084559) (198). PCR amplification of the no-template control and reverse transcription negative control (Figure 5-6 A and B) indicates that there was no contaminating DNA. Also shown is a typical real time PCR trace showing the amplification of D6D and cyclophilin A (Figure 5-7). The C_T value of D6D was compared with that of cyclophilin A to normalise the expression of D6D between samples (ΔC_T).

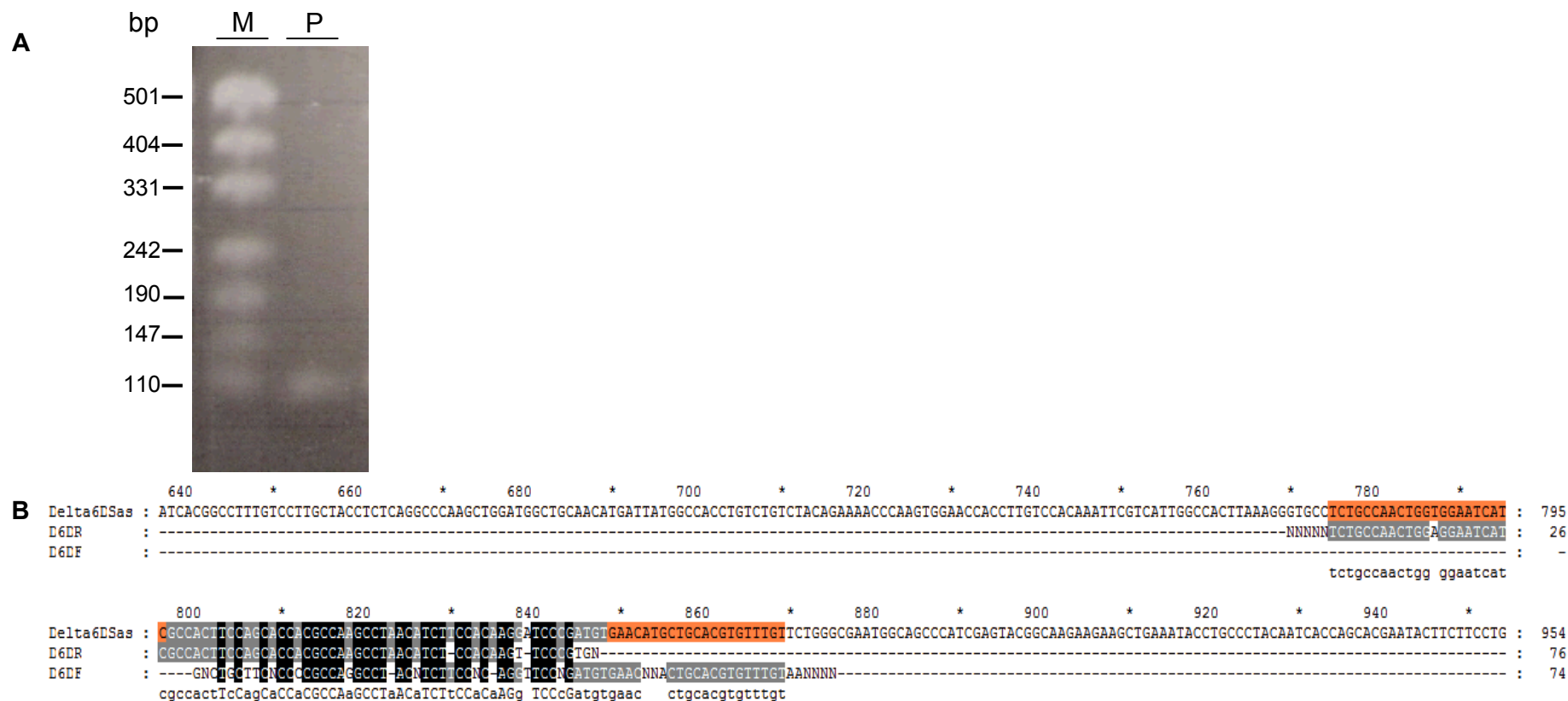


Figure 5-5. Agarose gel electrophoresis of the $\Delta 6$ desaturase PCR product (93 bp). M; marker, P; PCR product. (B) Sequence alignment of $\Delta 6$ desaturase reverse (D6DR) and forward (D6DF) PCR products. Delta6DSas is the published sequence of $\Delta 6$ desaturase (198). The areas shaded in orange are the forward and reverse primers. The areas shaded in grey indicate the alignment between the PCR product sequence and the published sequence. Line four is the consensus sequence; bases written in lower case indicate that either the reverse or forward sequence correlates with the published sequence. Bases written in capital letters indicate that both the reverse and forward sequence correlated with the published sequence.

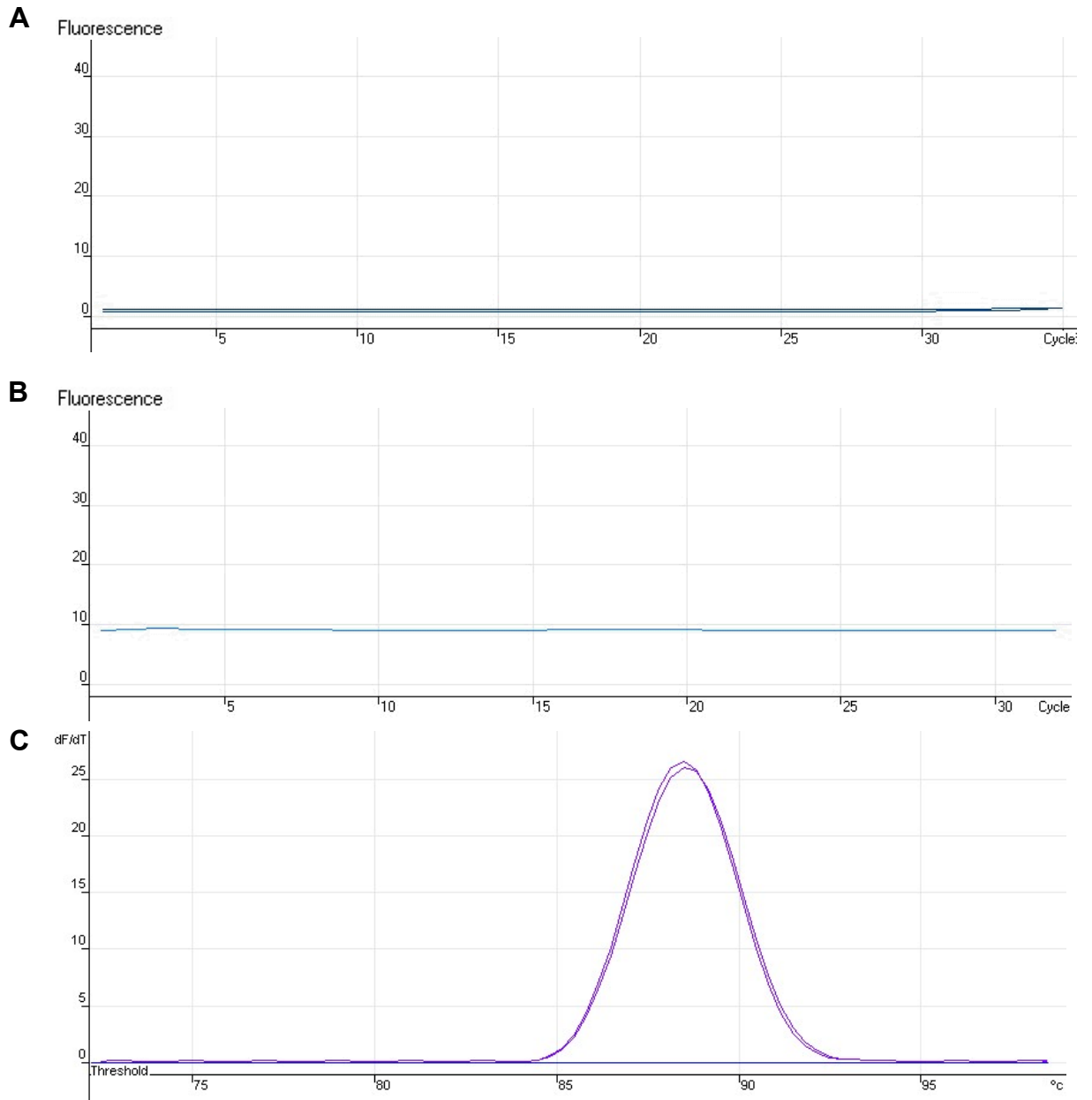


Figure 5-6. No template control, reverse transcription (RT) negative and melt curve analysis of the $\Delta 6$ desaturase PCR product using cDNA reverse transcribed from RNA isolated from HepG2 cells as a template.

(A). No template control. (B) RT negative control (C) Melt curve analysis of PCR product showing a single melting peak.

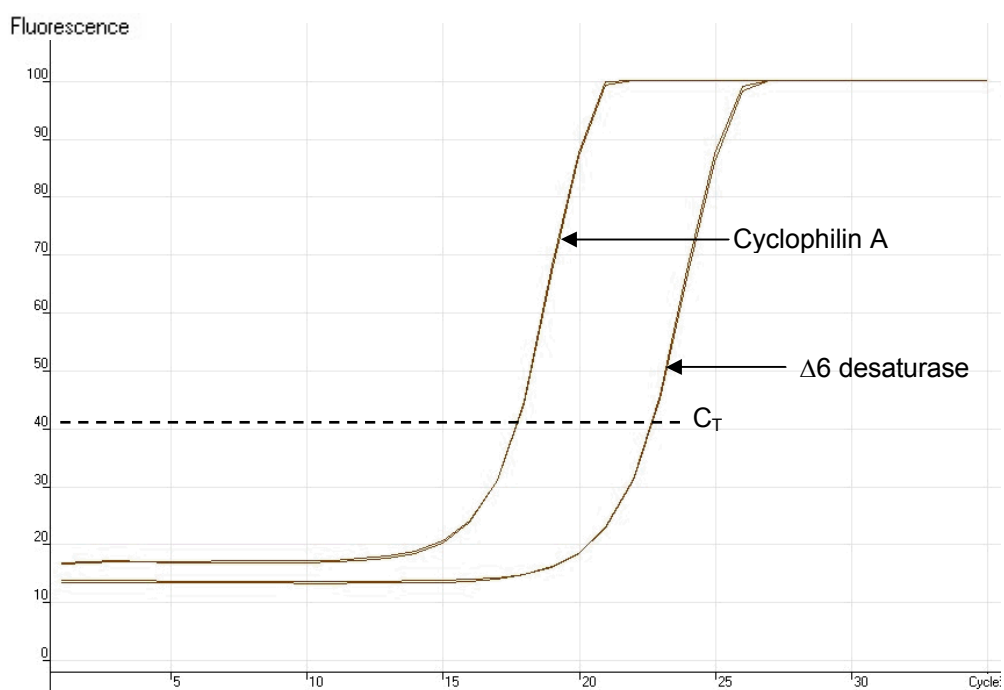


Figure 5-7. Amplification analysis of the housekeeping gene (cyclophilin A) and $\Delta 6$ desaturase gene using cDNA reverse transcribed from RNA isolated from HepG2 cells as a template (C_T ; cycle threshold).

5.3.2 $\Delta 6$ desaturase mRNA abundance

The effect of fatty acid supplementation on the mRNA abundance of D6D in HepG2 cells was assessed by quantitative RT-PCR. In the presence of 20 $\mu\text{g/ml}$ of OA, LA, ALA, AA or EPA the abundance of D6D mRNA was significantly reduced by $39 \pm 6.6\%$, $40 \pm 2.2\%$, $31 \pm 5.2\%$, $55 \pm 4.8\%$ and $52 \pm 5.0\%$, respectively (Figure 5-8), compared with control cells maintained in serum-free media with BSA. The suppression of D6D mRNA was not specific to any particular class of fatty acid as all fatty acids tested suppressed D6D mRNA abundance ($p < 0.05$). Supplementation with the LCPUFA, AA and EPA, appeared to suppress the expression of D6D mRNA more so than the 18 carbon fatty acids, although this reduction was not statistically significant ($p > 0.05$).

At time course on the effect of ALA supplementation (20 $\mu\text{g/ml}$) on the abundance of D6D mRNA over a 48 h supplementation period was also examined (Figure 5-9). D6D mRNA abundance was significantly reduced after 24 h supplementation with ALA. However, at 48 h, the time at which the medium was refreshed, the D6D mRNA abundance was equivalent to that observed in cells harvested at 0 h or maintained in serum-free medium for 48 h. The fatty acid composition of HepG2 cells supplemented with 20 $\mu\text{g/ml}$ ALA over the 48 h supplementation period was described in Chapter 3 (Figure 3-4).

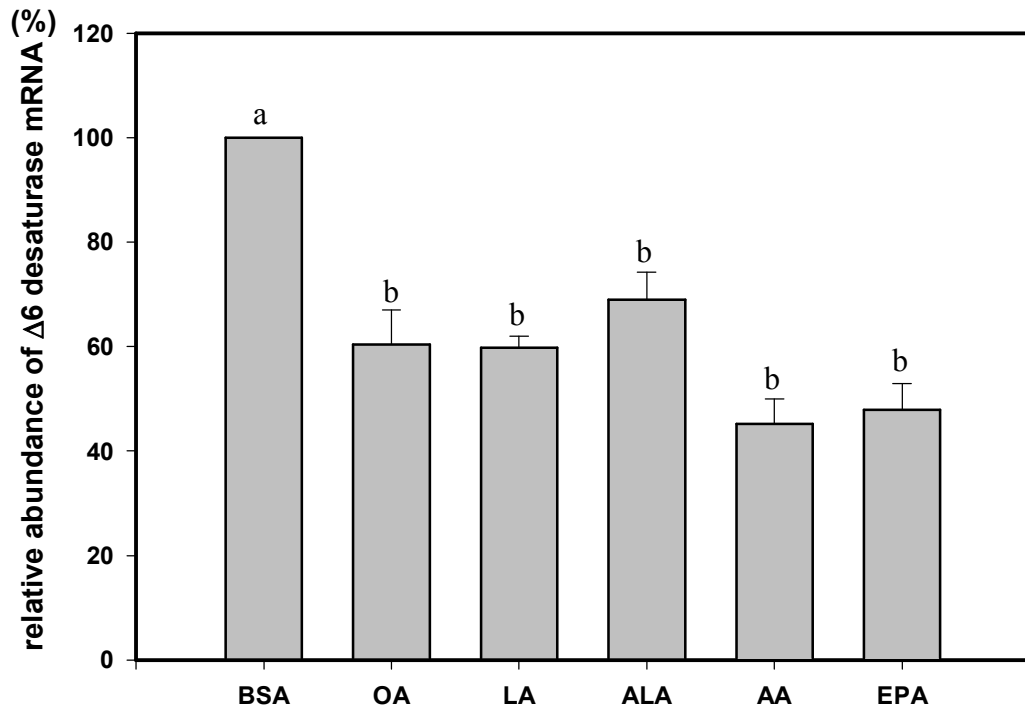


Figure 5-8. Relative abundance of $\Delta 6$ desaturase mRNA compared with the housekeeping gene, cyclophilin A, in HepG2 cells following supplementation with 20 μ g/ml oleic acid (18:1n-9, OA), linoleic acid (18:2n-6, LA), α -linolenic acid (18:3n-3, ALA), arachidonic acid (20:4n-6, AA) and eicosapentaenoic acid (20:5n-3, EPA).

HepG2 cells were seeded and grown as described. After 72h the medium was replaced with serum-free medium containing BSA-bound fatty acids for a further 48 h, as described in materials and methods. The cells were harvested for RNA extraction and real-time quantitative PCR analysis. Values are means \pm SE of at least five replicates. Values with different symbols are significantly different ($p < 0.05$) from each other by one-way ANOVA with Bonferroni post-hoc analysis.

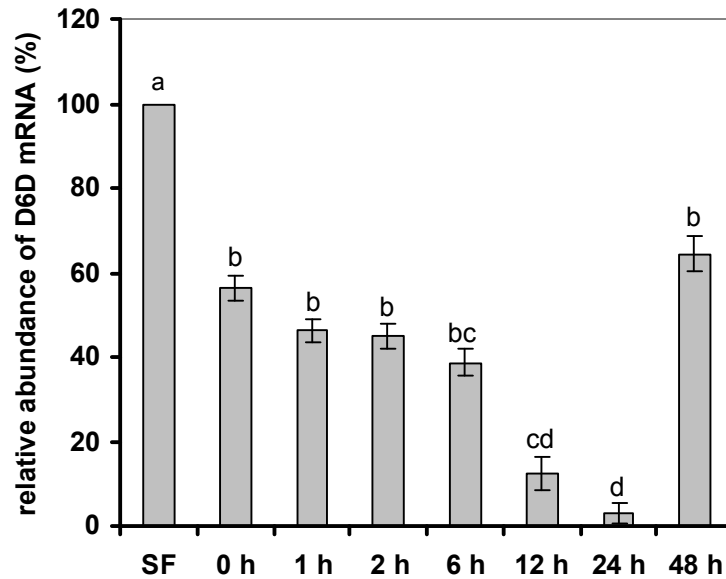


Figure 5-9. Relative abundance of $\Delta 6$ desaturase mRNA to the housekeeping gene, cyclophilin A, in HepG2 cells immediately prior to supplementation (0 h) and then at 1, 2, 6, 12, 24 and 48 h after supplementation with 20 $\mu\text{g/ml}$ α -linolenic acid (18:3n-3, ALA).

HepG2 cells were seeded and grown as described. After 72 h the medium was replaced with serum-free medium containing 20 $\mu\text{g/ml}$ ALA bound to BSA. The cells were harvested for RNA extraction and real-time quantitative PCR analysis immediately prior to supplementation with ALA (0 h), and then at 1, 2, 6, 12, 24 and 48 h after supplementation. SF; cells maintained in serum-free medium for 48 h. Values are means \pm SE of at least five replicates. Values with different symbols are significantly different ($p < 0.05$) from each other by one-way ANOVA with Bonferroni post-hoc analysis.

5.3.3 $\Delta 6$ desaturase protein expression

To determine whether the mRNA abundance of D6D correlated with the protein level of D6D, Western blot analysis of D6D protein in HepG2 cells supplemented with fatty acids was performed. Figure 5-10 shows the Western blot analysis of total cell protein lysates from HepG2 cells supplemented with the fatty acids OA, EPA, AA, ALA or LA or maintained in serum free media. The previous use of the anti-rat D6D antibodies in COS-7 cells transfected with rat D6D revealed a single band at 45 kDa (95). In the HepG2 cells used here, Western blot analysis of HepG2 cell protein did not detect any bands at 45 kDa but detected five bands at approximately 30, 35, 60, 78 and 100 kDa. Although the D6D protein could not be clearly identified, the expression of these bands was consistent between cells supplemented with OA, LA, ALA, AA and EPA and cells maintained in serum-free media.

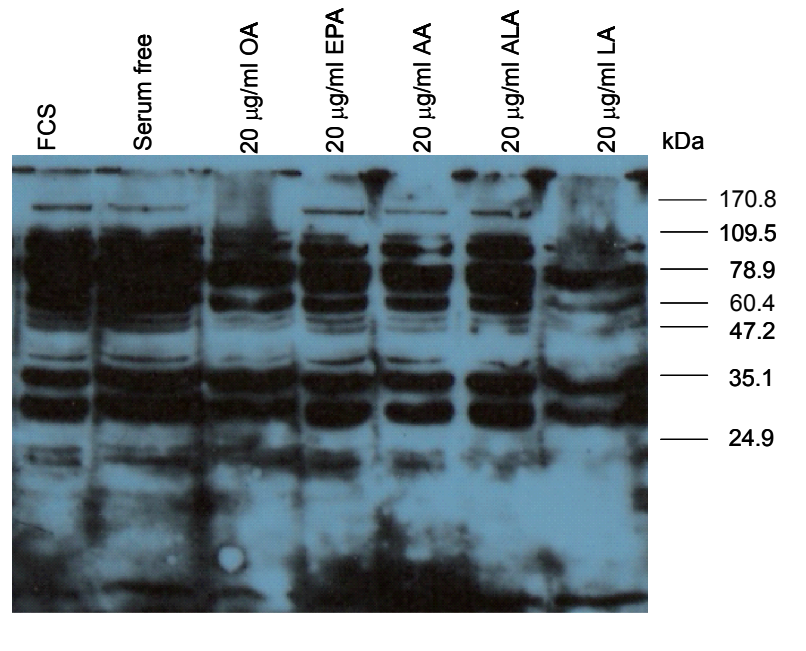


Figure 5-10. Detection of $\Delta 6$ desaturase in HepG2 cells supplemented with 20 $\mu\text{g/ml}$ oleic acid (18:1n-9, OA), eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, AA), α -linolenic acid (18:3n-3, ALA) or linoleic acid (18:2n-6, LA) bound to BSA in serum free DMEM or maintained in DMEM + 10% FBS (FBS) or serum free DMEM (serum free) for 48 h. Total cell protein lysates (50 μg) were resolved by SDS-PAGE and blotted. The blot was probed with anti-rat $\Delta 6$ desaturase sera (serum 1 and 2) at 1:1000 dilution. Detection was performed using chemifluorescence after 30 s. Molecular masses are indicated in kDa.

5.3.4 Fatty acid analysis

Despite the reduction in D6D mRNA abundance by all fatty acids examined, the concentration of D6D conversion products (20:3n-9, AA and EPA), in respective OA, LA and ALA supplemented cells were elevated above that observed in control cells (Table 5-1). There was a significant increase in the level of OA and its product, 20:3n-9, in cells supplemented with 20 µg/ml OA. In cells supplemented with 20 µg/ml OA, the level of OA in cell phospholipids increased from $20.69 \pm 0.52\%$ total fatty acids in control cells to $30.74 \pm 0.66\%$ total fatty acids and the level of 20:3n-9 increased from $5.36 \pm 0.36\%$ total fatty acids to $7.37 \pm 0.19\%$ total fatty acids. The level of n-3 and n-6 fatty acids was unchanged in cells supplemented with OA compared with control cells (Table 5-1). The fatty acid composition of HepG2 cells supplemented with 20 µg/ml LA and 20 µg/ml ALA has been described in (Section 3.3.4) and (Section 3.3.1), respectively. Following supplementation with 20 µg/ml AA, the level of AA in HepG2 cell phospholipids increased dramatically, as did the level of 22:5n-6, which increased 10-fold from $0.17 \pm 0.01\%$ in control cells to $1.73 \pm 0.10\%$ total fatty acids. The total level of monounsaturated and n-9 fatty acids were reduced, however, the total level of n-3 fatty acids was unchanged compared with control cells (Table 5-1). In HepG2 cells supplemented with 20 µg/ml EPA, the level of EPA in cell phospholipids increased from $0.14 \pm 0.02\%$ total fatty acids to $14.95 \pm 0.51\%$ total fatty acids. The level of DPA and DHA also increased significantly ($p < 0.05$), increasing from $0.24 \pm 0.06\%$ total fatty acids and $2.61 \pm 0.08\%$ total fatty acids in control cells to $5.38 \pm 0.21\%$ total fatty acids and $4.48 \pm 0.13\%$ total fatty acids, respectively (Table 5-1)

Fatty acid	BSA control	20µg/ml OA	20µg/ml EPA	20µg/ml AA	20µg/ml ALA	20µg/ml LA
Total Saturates	29.63 ± 0.18 ^a	28.05 ± 0.32 ^a	38.57 ± 0.60 ^{c,d}	36.31 ± 0.62 ^{b,d}	35.29 ± 1.44 ^{b,c}	34.55 ± 0.30 ^b
18:1n-9 OA	20.69 ± 0.52 ^a	30.74 ± 0.66 ^b	10.16 ± 0.66 ^c	9.94 ± 0.68 ^c	11.23 ± 0.75 ^c	9.86 ± 0.87 ^c
Total Monounsaturates	51.99 ± 0.27 ^a	48.42 ± 0.29 ^a	29.26 ± 0.26 ^{b,c}	29.57 ± 0.19 ^{b,c}	32.34 ± 1.60 ^c	26.92 ± 1.67 ^b
20:3n-9	5.36 ± 0.36 ^a	7.37 ± 0.19 ^b	1.85 ± 0.00 ^c	1.92 ± 0.03 ^c	2.24 ± 0.21 ^c	2.12 ± 0.19 ^c
Total n-9	33.19 ± 0.31 ^a	44.96 ± 0.57 ^b	15.28 ± 0.72 ^c	15.71 ± 0.72 ^c	17.56 ± 1.20 ^c	15.02 ± 1.22 ^c
18:2n-6 LA	0.73 ± 0.06 ^a	0.59 ± 0.03 ^a	0.61 ± 0.02 ^a	0.63 ± 0.03 ^a	0.86 ± 0.01 ^a	15.92 ± 0.24 ^b
18:3n-6	0.11 ± 0.03 ^a	0.08 ± 0.00 ^a	0.06 ± 0.03 ^a	0.10 ± 0.05 ^a	0.09 ± 0.02 ^a	0.43 ± 0.01 ^b
20:3n-6	0.48 ± 0.02 ^a	0.36 ± 0.01 ^a	0.34 ± 0.03 ^a	0.47 ± 0.08 ^a	0.43 ± 0.01 ^a	2.83 ± 0.20 ^b
20:4n-6 AA	2.94 ± 0.14 ^a	2.56 ± 0.07 ^a	2.39 ± 0.03 ^a	22.53 ± 0.65 ^c	2.64 ± 0.08 ^a	10.60 ± 0.83 ^b
22:5n-6	0.17 ± 0.01 ^a	0.16 ± 0.01 ^a	0.07 ± 0.00 ^a	1.73 ± 0.10 ^c	0.14 ± 0.03 ^a	1.19 ± 0.17 ^b
Total n-6	4.78 ± 0.28 ^a	3.80 ± 0.12 ^a	3.51 ± 0.11 ^a	27.83 ± 0.73 ^c	4.40 ± 0.13 ^a	32.22 ± 1.70 ^b
18:3n-3 ALA	0.11 ± 0.13 ^a	0.01 ± 0.01 ^a	0.03 ± 0.00 ^a	0.02 ± 0.02 ^a	7.05 ± 0.53 ^b	0.05 ± 0.00 ^a
20:5n-3 EPA	0.14 ± 0.02 ^a	0.08 ± 0.00 ^a	14.95 ± 0.51 ^c	0.04 ± 0.01 ^a	7.07 ± 0.74 ^b	0.07 ± 0.01 ^a
22:5n-3 DPA	0.24 ± 0.06 ^a	0.30 ± 0.01 ^a	5.38 ± 0.21 ^c	0.23 ± 0.02 ^a	1.91 ± 0.31 ^b	0.17 ± 0.01 ^a
22:6n-3 DHA	2.61 ± 0.08 ^a	2.14 ± 0.06 ^{a,c}	4.48 ± 0.13 ^b	1.48 ± 0.14 ^c	3.93 ± 0.22 ^b	2.11 ± 0.06 ^a
Total n-3	3.95 ± 0.29 ^a	2.53 ± 0.07 ^a	25.35 ± 0.39 ^b	2.35 ± 0.07 ^a	22.69 ± 1.44 ^b	2.75 ± 0.07 ^a

Table 5-1. Fatty acid composition of HepG2 cell phospholipids of cells supplemented 20µg/ml oleic acid (18:1n-9, OA), eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, AA), α -linolenic acid (18:3n-3, ALA) and linoleic acid (18:2n-6, LA). HepG2 cells were cultured as described in Materials and Methods and supplemented with for 20µg/ml OA, LA, ALA, AA and EPA 48 h. Values are means ± SE of at least three replicates. Values with different superscripts are significantly different from each other (p < 0.05).

5.4 DISCUSSION

The mRNA abundance of D6D in HepG2 cells was significantly reduced following supplementation with OA, EPA, AA, ALA or LA compared with cells maintained in serum-free media. However, the accumulation of fatty acid conversion products suggests that the activity of the D6D protein may not be reduced despite a report that showed the presence of these fatty acids suppressed D6D mRNA abundance (107). Despite a reduction in D6D mRNA abundance by up to 50%, the accumulation of fatty acid conversion productions was increased compared with control cells.

Western blot analysis of HepG2 cell protein did not specifically identify D6D, suggesting non-specific binding of the polyclonal antibodies. D6D antibodies were not commercially available at the time of this publication, however two research groups have developed an antibody. I was able to acquire antibodies raised against the rat D6D developed by Professor Philippe Legrand and colleagues (95). Due to the limited availability of a human D6D antibody, a rat antibody was used to examine D6D protein expression in a human cell line. The rat and human D6D share 95.5% amino acid sequence identity and both are 444 amino acids in length. The sequence identity over the region that the primary antibodies, serum 1 and serum 2, were raised was 92.5 and 85.5%, respectively. D'Andrea *et al.* (2002) (95) performed Western Blot analysis of the total homogenate obtained from COS-7 cells transfected with the full length (1335 bp) open reading frame of rat D6D and detected a single band at 45 kDa. This band was not detected in non-transfected cells. In a previous study, Western blot analysis of rat microsomes using an affinity-purified anti-rat D6D antibody identified a protein at 52.2 kDa (115). Another study predicted a protein size of 52.2 kDa for the human D6D from the open reading frame of the enzyme (105). A band of this size was evident in all cells regardless of fatty

acid treatment (Figure 5-10). D'Andrea *et al.* (2002) (95) also reported that Western blot analysis could not specifically detect the D6D protein in protein isolated from rat liver or brain and required immunoprecipitation of the isolated protein with both anti-rat D6D sera prior to Western blot analysis. This suggests that a significant enrichment of D6D protein would be necessary before Western blot analysis with these antibodies. The detection of multiple bands in the Western blot analysis of HepG2 whole cell protein lysates suggests immunoprecipitation of the whole cell protein lysates may help to resolve the specific band from the multiple bands. The detection system used in the experiments described here (ECL Super Signal West Femto Kit, Pierce, Rockford, IL, USA) was apparently up to 1000 times more sensitive than the system used by D'Andrea *et al.* (2002) (95) (ECL Plus, GE Healthcare, Piscataway, NJ, USA), detecting several bands in the Western blot analysis of HepG2 cell protein lysates. Given the apparent sensitivity of the detection system, the results suggest that the antibodies may have required further purification for use under these conditions. A positive control for the Western blot using recombinant protein would assist in determining the size of the D6D protein under these conditions. In addition, pre-bleed sera to assess any non-specific binding of the anti-rat D6D antibody in the HepG2 preparations would help to identify D6D, although D'Andrea *et al.* (2002) (95) reported that no bands were detected with pre-immune sera. It is possible that the multiple bands observed in Figure 5-10 may be due to splice variants of D6D or post-translational modification of the protein isolated from HepG2 cells. However, without the appropriate controls it is difficult to make any clear conclusions from this data. Future work using these anti-rat D6D antibodies should assess the expression in immunoprecipitates of HepG2 whole cell lysates. Although D6D mRNA expression was reduced by as much as 50%, the conversion of substrate fatty acids to their long chain metabolites was evident.

Further research is required to determine whether this relates to the level of D6D protein and its activity.

Dietary PUFA is reportedly a major regulator of D6D mRNA expression (88) which may influence the accumulation of LCPUFA in cell membranes. The suppression of D6D mRNA abundance by PUFA has been described previously in human neuroblastoma cells (181) and HepG2 cells (107). However, such studies did not correlate D6D mRNA expression with fatty acid composition. Nara *et al.* (2002) (164) showed that the abundance of D6D mRNA was reduced by approximately 50% in HepG2 cells supplemented with AA, EPA and DHA for 24 h whereas OA had no effect. The results presented here for D6D mRNA expression following supplementation with AA and EPA are in agreement with Nara *et al.* (2002) (164), however, the difference in the effect of OA may be due to their inclusion of insulin and dexamethasone, two hormones that influence the expression of PPAR (202) and indeed, D6D mRNA expression (203). The conditions under which cells are cultured, as well as the level of fatty acids, may modulate D6D mRNA abundance by affecting the transcription rate of the D6D gene or the stability of the mRNA. The mRNA expression of D6D following supplementation with ALA over 48 h suggests that the cell culture conditions may influence D6D mRNA expression as there was a significant change in expression between 24 and 48 h, the time at which the media was refreshed (Figure 5-9). Following 24 h supplementation with ALA, the expression of D6D mRNA was less than 5% of control cells. However, at 48 h, the expression of D6D mRNA was less than 70% of controls. The change in D6D gene expression over the ALA-supplementation period suggests that a time course for each supplemented fatty acid may be required in identifying the maximum reduction in D6D mRNA expression in future studies.

Previous *in vivo* studies have demonstrated that dietary OA does not affect D6D mRNA expression (80;105;107;204), however the results present here indicate that not only do LA, ALA, AA, EPA suppress D6D mRNA abundance, but so to does OA, to similar levels as observed for PUFAs. That being said, other mechanisms *in vivo* may influence the expression of D6D including the metabolic state of the animal, which was not a factor in *in vitro* studies. For example, by examining the effect of one fatty acid on the expression of D6D mRNA, the diet is devoid of the essential fatty acids. Comparisons between animals maintained on dietary PUFA with those fed OA or 18:0 as a source of dietary fat may be more representative of the effects of essential fatty acid deficiency on D6D mRNA expression rather than a direct effect of dietary fatty acids on this expression.

Altered expression of D6D may also result due to mechanisms not directly related to fatty acid metabolism. In rats supplemented with the PPAR α agonist WY14643, the fatty acid oxidation enzymes, acyl CoA oxidase, L-bifunctional protein and cytochrome P450 4A1 were all significantly induced within 4 h of treatment, with maximum induction occurring at 28 h (205). D6D mRNA abundance was not significantly elevated at 4 h and it was only after 28 h that significance was reached (205). The increased expression of D6D mRNA after 28 h suggests that its induction was secondary to the induction of the oxidation enzymes by WY1463 and may be compensatory. Interestingly, the level of OA in liver phospholipids was elevated (205). OA is synthesised *de novo* from the saturated fatty acids, palmitic acid (16:0) or 18:0, usually in essential fatty acid deficient states. Moreover, PPAR agonists induce peroxisome proliferation in rodents (206). The changes in fatty acid metabolism may also be attributable to the increased demand for fatty acids as

constituents of phospholipids in the expanding peroxisome population (114). It is questionable whether the changes in the fatty acid composition of cell membranes is directly attributable to the increased mRNA expression of D6D by WY14643 but rather the increased demand for fatty acid oxidation as a result of PPAR activation. The effect of D6D mRNA expression on the fatty acid composition of cell membranes is still unclear.

The experimental conditions employed here to examine the effect of fatty acid supplementation on D6D mRNA expression in HepG2 cells may also alter the metabolic demands of the cells. Cells maintained in serum-free media without fatty acid supplementation had a reduced amount of substrate that could be utilised as a source of energy. The expression of D6D mRNA in 'control' cells may be induced in response to a reduction in energy. Under these conditions, the HepG2 cells may have an increased oxidative capacity therefore, it would be useful to examine enzymes involved in oxidation concurrent with D6D expression.

In vitro studies indicate that the mRNA expression of D6D is modulated by fatty acids to some extent. The fatty acid composition of cell membranes may be influenced by various parameters and highlights the need for a holistic approach to examining D6D mRNA expression, to include the expression of oxidation enzymes, enzyme activity and fatty acid composition. Further studies that examine the effect of D6D overexpression by transfection of a D6D expression vector, similar to that described by D'Andrea *et al.* (2002) (95), or the effect of knockdown of D6D mRNA by short interfering RNA, on the fatty acid composition of cell membranes may help in describing a relationship between the fatty acid composition and D6D expression.

6 Fatty acid composition of HepG2 cells treated with SC-26196

6.1 INTRODUCTION

In addition to the competition between substrate fatty acids for D6D, the fatty acid composition of cell lipids may also be affected by the preferential use of substrate fatty acids for incorporation into cell phospholipids, peroxisomal β -oxidation and/or conversion to LCPUFA (151;207). Intermediary fatty acids in the synthesis of DHA from n-3 fatty acid precursors do not appear in tissues in equal amounts, which may be due to the preferential entry of individual fatty acids into one of these pathways. In Chapter 4, the accumulation of DHA into HepG2 cells supplemented with increasing concentrations of DPA was curvilinear, yet the accumulation of its retroconversion product, EPA, was directly related to the concentration of DPA in the media, suggesting that peroxisomal β -oxidation of DPA to EPA may divert the conversion of DPA away from the synthesis DHA. For example, in peroxisomes incubated with [3-¹⁴C] DPA, in the presence of microsomes and the acyl acceptor, 1-acyl-GCP, the retroconversion product of DPA, EPA, was transport out of the peroxisomes for use in membrane lipid synthesis rather than for continued peroxisomal degradation (132). Moreover, in cultured skin fibroblasts, 24:6n-3 is preferentially oxidised rather than acting as a substrate for incorporation into membrane lipids (133). The relative entry of substrate fatty acids into phospholipid biosynthesis or peroxisomal β -oxidation will determine the availability of fatty acids for conversion to LCPUFA and represents a mechanism by which the fatty acid composition of cell membranes may be regulated.

This chapter aimed to examine the change in the accumulation of LCPUFA in HepG2 cells supplemented with DPA following treatment with the specific D6D

inhibitor 2,2-diphenyl-5-(4-(8)piperazine-1-yl)pentanenitrile (SC-26196). SC-26196 is a reputed highly selective inhibitor of D6D (208). The selectivity of the inhibitor for D6D was assessed by examining the accumulation of LCPUFA in HepG2 cells supplemented with the immediate D6D fatty acid substrate, ALA, and the D5D fatty acid substrate, 20:3n-6. In the mouse carrageenan paw oedema model of inflammation, SC-26196 reduced paw oedema by 50%, an effect directly linked to its ability to inhibit the conversion of LA to AA (208). The use of SC-26196 allowed the elongation of DPA to 24:5n-3, and its retroconversion to EPA, to be examined without becoming a substrate for D6D. This chapter describes the use of SC-26196 to confirm the involvement of D6D in the conversion of fatty acids in HepG2 cells and the fatty acid compositional changes which occur in cell phospholipids as a result of D6D inhibition.

6.2 MATERIALS AND METHODS

The materials and methods used were those described in Chapter 2, with the following changes.

6.2.1 Materials

SC-26196 was kindly donated by Pfizer Inc. (Groton, CT, USA) (208). DMSO was from Sigma (St. Louis, MO, USA).

6.2.2 Cell Culture

HepG2 cells were cultured and seeded into 6 well plates as described in Section 3.2.1. After 72 h, the medium was removed and replaced with serum-free DMEM supplemented with 5 µg/ml ALA, DPA or 20:3n-6, bound to fatty acid-free BSA. Stock solutions of these fatty acids were prepared in ethanol at a concentration of 10 mg/ml and diluted in serum-free medium to achieve a final concentration of 5 µg/ml.

As described in earlier chapters, the maximal accumulation of DHA in HepG2 cell phospholipids following supplementation with increasing concentrations of n-3 fatty acids occurred at 5 µg/ml. Therefore, the effect of SC-26196 on the fatty acid composition of cells supplemented with 5 µg/ml 20:3n-6, ALA or DPA was examined. In experiments using SC-26196, the medium was replaced at 72 h with either SC-26196, dissolved in DMSO, to yield a final concentration of 2 µM, or an equivalent volume of DMSO alone, in addition to 20:3n-6, ALA or DPA at 5 µg/ml.

In human skin fibroblasts, 2 µM SC-26196 has been shown to reduce the conversion of ALA to EPA, DPA and DHA by 80% (209), therefore the same concentration of SC-26196 was used here. The media was refreshed after 24 h with the appropriate concentration of fatty acid bound to BSA with SC-26196 as appropriate. Cells were harvested for fatty acid analysis following a total 48 h supplementation period.

6.2.3 Cytotoxicity assay

Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release from cells using the CytoTox 96® Non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA). This assay quantitatively measures LDH, a stable cytosolic enzyme that is released upon cell lysis by a coupled enzymatic reaction that converts a tetrazolium salt (INT) to a red formazan product. The amount of colour formed is proportional to the number of lysed cells. To assess the cytotoxicity of SC-26196, cells were seeded in 96-well plates at 0.04×10^6 cells / well in 100 µL growth medium. After 72 h, the medium was removed and the cells were washed twice with PBS. Cells in quadruplicate wells were incubated in serum-free DMEM supplemented with 5 µg/ml ALA or DPA, bound to essentially fatty acid-free BSA ± SC-26196 or the appropriate controls. The media was refreshed after 24 h as

described in Section 6.2.2. At the end of fatty acid supplementation, 50 μ L of the cell supernatant was transferred to a fresh 96 well enzymatic assay plate (Greiner Bio One, Frickenhausen, Germany) and processed according to the manufacturers instructions (Promega, Madison, WI, USA) The absorbance of the coloured formazan product was measured on an automatic microplate reader at an optical density of 490 nm using Genesis-Lite software (Labsystems and Life Sciences International, Hampshire, UK). The percent cytotoxicity was determined by dividing the absorbance of experimental cells by the maximum absorbance, multiplied by 100.

6.2.3.1 Cell Viability and cytotoxicity

The viability and number of cells following treatment with SC-26196 was not significantly different to untreated cells, and supplementation with either ALA or DPA did not effect the number or viability of HepG2 cells (Figure 6-1A &B). There was no significant cytotoxicity associated with SC-26196 (Figure 6-2).

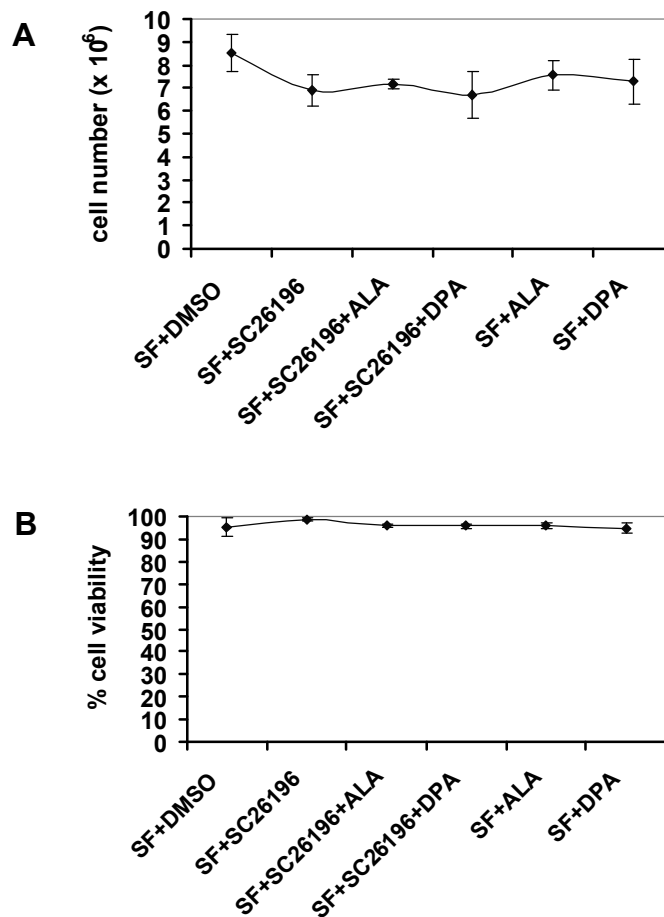


Figure 6-1. Cell number (A) and viability (B) of HepG2 cells cultured in serum-free DMEM (SF) supplemented with α -linolenic acid (18:3n-3, ALA) or docosapentaenoic acid (22:5n-3, DPA) and treated with the Δ 6 desaturase inhibitor, SC-26196.

Viability was determined by trypan blue exclusion as described in Section 2.2.5.

There were no significant differences ($p > 0.05$) in cell number or viability between groups using One-way ANOVA with Bonferroni post-hoc tests.

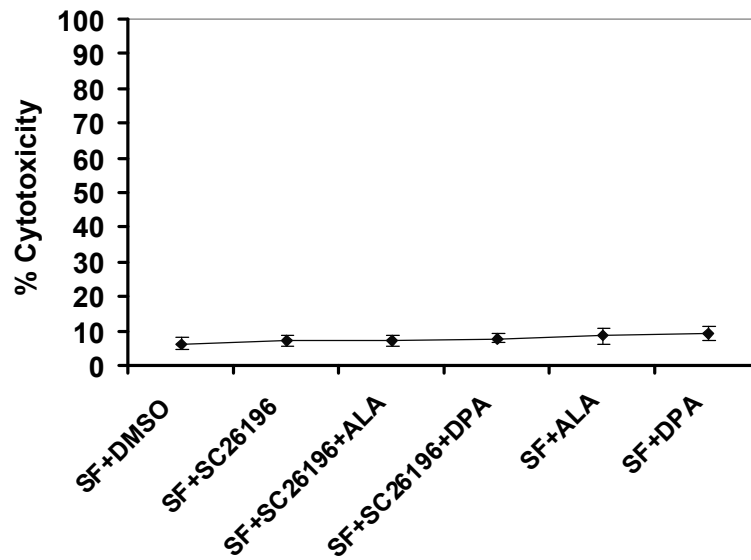


Figure 6-2. Cytotoxicity of SC-26196 in HepG2 cells.

HepG2 cells were cultured in serum-free DMEM (SF) supplemented with α -linolenic acid (18:3n-3, ALA) or docosapentaenoic acid (22:5n-3, DPA) and treated with the $\Delta 6$ desaturase inhibitor, SC-26196. Cytotoxicity of SC-26196 in HepG2 cells was determined by measuring lactate dehydrogenase (LDH) release from cells using the CytoTox 96® Non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) as described in Section 6.2.3. There were no significant differences ($p > 0.05$) in cytotoxicity between groups using One-way ANOVA with Bonferroni post-hoc tests.

6.2.4 Fatty acid analysis

Total lipids were extracted from cells following procedures described in Chapter 2.

6.2.5 Statistical analysis

Statistical analyses were performed on at least three separate replicates. Independent Student-T tests were used to compare means following fatty acid supplementation, with or without SC-26196 treatment, for values that were normally distributed otherwise the Nonparametric Mann-Whitney U tests were used. Statistical tests were performed using the SPSS Statistical Software Package Version 11.0 (SPSS Inc

Chicago, IL, USA). Statistical significance was defined as $p < 0.05$. Data are expressed as mean \pm SE.

6.3 RESULTS

6.3.1 Validation of the action of SC-26196

To confirm the selective action of SC-26196 in inhibiting D6D, the effect of SC-26196 on the fatty acid composition of HepG2 cells supplemented with the D6D substrate, ALA, and the D5D substrate, 20:3n-6, respectively, was examined. In untreated HepG2 cells supplemented with 5 μ g/ml ALA, the major products of ALA metabolism were EPA and DHA (Figure 6-3). Treatment of ALA-supplemented HepG2 cells with 2 μ M SC-26196 reduced the level of EPA, DPA and DHA by 92.87%, 62.41% and 39.53%, respectively. A 4-fold increase in the accumulation of ALA ($p < 0.05$) in HepG2 cell phospholipids was observed (Figure 6-3). There was also a significant reduction ($p < 0.05$) in the accumulation of 24:5n-3 and 24:6n-3 in ALA-supplemented cells treated with SC-26196. ALA-supplemented cells treated with SC-26196, accumulated significantly higher ($p < 0.05$) quantities of the direct elongation product of ALA, 20:3n-3, which increased from $0.53 \pm 0.13\%$ total fatty acids in untreated ALA-supplemented cells to $1.58 \pm 0.25\%$ total fatty acids in treated cells. The level of 20:3n-3 is usually present in trace amounts in HepG2 cells supplemented with ALA due to the activity of D6D which converts ALA to 18:4n-3 to continue through the desaturation-elongation pathway (Table 6-1). In SC-26196 treated cells this does not occur.

As demonstrated in a previous chapter (see Chapter 3, Table 3-1) the level of saturated fatty acids in untreated HepG2 cell phospholipids increased and the level of monounsaturated fatty acids decreased in cells supplemented with increasing

concentrations of ALA. In contrast, the proportion of monounsaturated fatty acids in cell phospholipids increased significantly ($p < 0.05$), concomitant with a decrease in the level of saturated fatty acids in HepG2 cells treated with 2 μM SC-26196 and supplemented with ALA. This may indicate an up-regulation in the conversion of saturated fatty acids to monounsaturated fatty acids by stearoyl-CoA desaturase (SCD) when D6D is inhibited by SC-26196 (168;210). The level of n-6 fatty acids in ALA-supplemented HepG2 cells was not affected by treatment with SC-26196. These results are summarised in Table 6-1. Treatment of HepG2 cells with SC-26196, without fatty acid supplementation, did not affect the fatty acid composition of cell phospholipids compared with HepG2 cells maintained in serum-free media (see appendix 2).

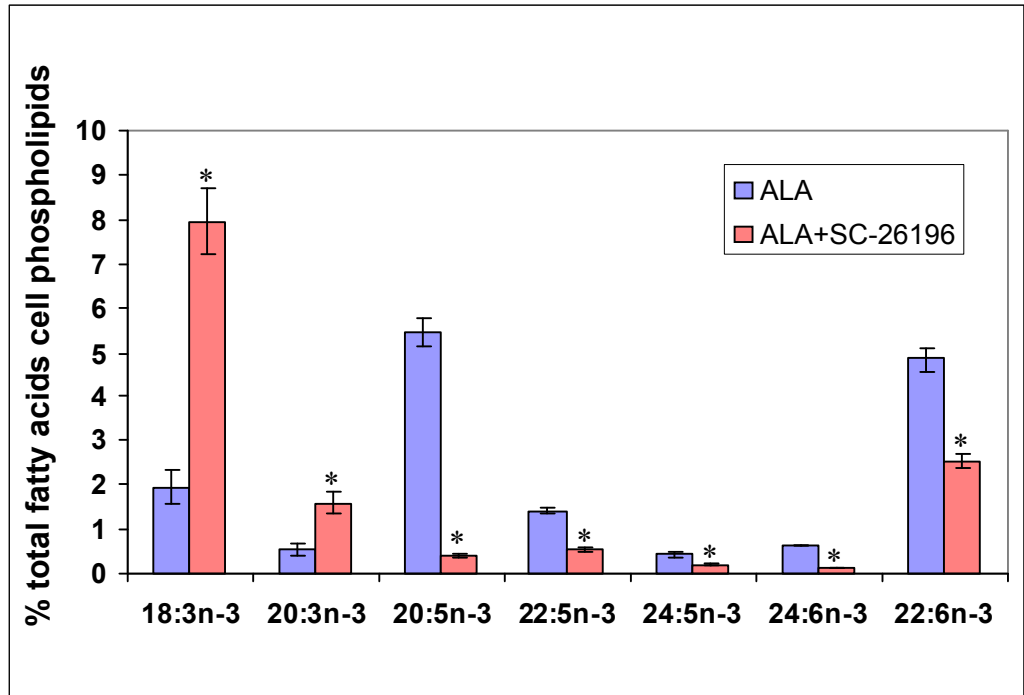


Figure 6-3. The accumulation of α -linolenic acid (18:3n-3, ALA) and its metabolites in HepG2 cells supplemented with 5 μ g/ml ALA alone or treated with the Δ 6 desaturase inhibitor, SC-26196.

HepG2 cells were cultured as described and supplemented with 5 μ g/ml ALA alone (blue) or with SC-26196 (pink) for 48 h.

* Indicates means are significantly different ($p < 0.05$) from untreated HepG2 cells supplemented with 5 μ g/ml ALA.

	5 µg/ml ALA	5 µg/ml ALA + 2 µM SC-26196
Fatty acid	% total fatty acids	
Total Saturates	34.53 ± 0.23	30.23 ± 0.30*
18:1n-9 OA	13.39 ± 0.13	15.93 ± 0.36*
Total Monounsaturates	41.89 ± 0.85	47.12 ± 1.44*
20:3n-9	1.66 ± 0.83	1.44 ± 0.70
Total n-9	18.21 ± 0.88	20.52 ± 0.97
18:2n-6 LA	0.87 ± 0.04	1.05 ± 0.06
18:3n-6	0.10 ± 0.02	0.14 ± 0.01
20:3n-6	0.47 ± 0.02	0.48 ± 0.02
20:4n-6 AA	2.68 ± 0.08	2.98 ± 0.14
22:5n-6 DPA n-6	0.16 ± 0.01	0.17 ± 0.004
Total n-6	4.55 ± 0.26	5.19 ± 0.33
18:3n-3 ALA	1.95 ± 0.36	7.95 ± 0.73*
20:3n-3	0.53 ± 0.13	1.58 ± 0.25
20:5n-3 EPA	5.47 ± 0.31	0.39 ± 0.04*
22:5n-3 DPAn-3	1.41 ± 0.07	0.53 ± 0.04*
24:5n-3	0.43 ± 0.07	0.20 ± 0.03*
24:6n-3	0.61 ± 0.003	0.13 ± 0.004*
22:6n-3 DHA	4.84 ± 0.29	2.53 ± 0.14*
Total n-3	16.21 ± 1.04	14.61 ± 1.11

Table 6-1. Fatty acid composition of cell phospholipids of HepG2 cells supplemented with 5 µg/ml α -linolenic acid (18:3n-3, ALA) alone or treated with the Δ 6 desaturase inhibitor, SC-26196.

HepG2 cells were cultured as described and supplemented with 5 µg/ml ALA alone or with SC-26196 for 48 h. Values marked with an asterisk are significantly different from untreated cells as determined by the Independent Student t-test ($p < 0.05$).

Values are means \pm SE.

6.3.1 Continued

The addition of SC-26196 to cells supplemented with 20:3n-6 did not affect the accumulation of AA (20:4n-6), indicating that SC-26196 did not inhibit D5D and was specific for D6D (Figure 6-4). These results are summarised in Table 6-2

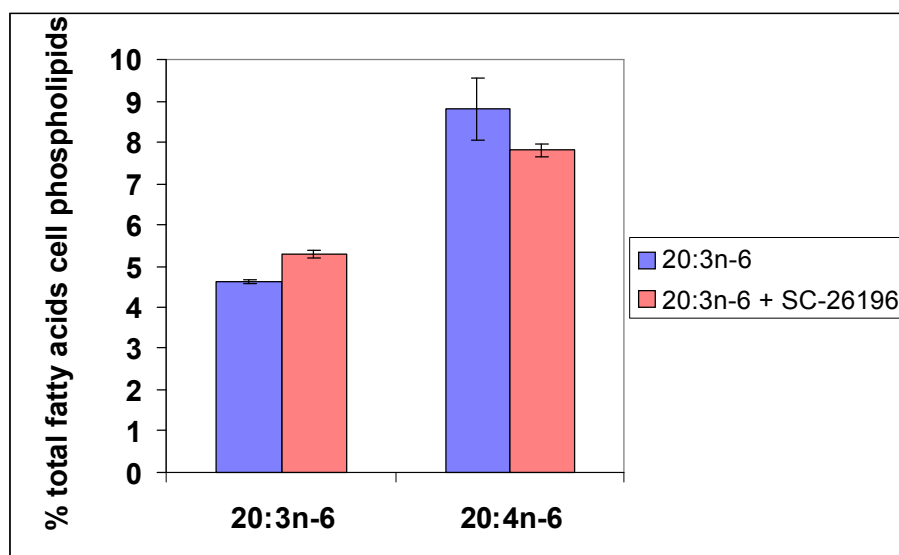


Figure 6-4. The accumulation of 20:3n-6 and arachidonic acid (20:4n-6, AA) in HepG2 cells supplemented with 5 μ g/ml 20:3n-6 alone or treated with the Δ 6 desaturase inhibitor, SC-26196.

HepG2 cells were cultured as described and supplemented with 5 μ g/ml 20:3n-6 alone (blue) or with SC-26196 (pink) for 48 h. There was no significant difference ($p < 0.05$) in the level of 20:3n-6 or its Δ 5 desaturation product, AA, in supplemented HepG2 cells treated with SC-26196.

	5 µg/ml 20:3n-6	5 µg/ml 20:3n-6 + 2 µM SC-26196
Fatty acid	% total fatty acids	
Total Saturates	34.18 ± 0.73	30.85 ± 0.69 ^{‡*}
18:1n-9	13.39 ± 0.12	15.54 ± 0.31 ^{‡*}
Total Monounsaturates	41.61 ± 0.25	46.89 ± 1.04 ^{‡*}
20:3n-9	3.66 ± 0.12	2.01 ± 0.02 ^{‡*}
Total n-9	21.16 ± 0.10	20.57 ± 0.39 [‡]
18:2n-6	0.68 ± 0.03	0.84 ± 0.04 ^{‡*}
18:3n-6	0.087 ± 0.01	0.14 ± 0.01 ^{‡*}
20:3n-6	4.66 ± 0.04	5.30 ± 0.76 [†]
20:4n-6	8.80 ± 0.08	7.82 ± 0.15 [†]
22:5n-6	0.66 ± 0.31	0.13 ± 0.05 [‡]
Total n-6	15.24 ± 0.39	14.69 ± 0.70 [‡]
18:3n-3		
20:5n-3	0.060 ± 0.00	0.087 ± 0.00 [†]
22:5n-3	0.28 ± 0.01	0.32 ± 0.06 [‡]
24:5n-3	0.050 ± 0.00	0.15 ± 0.00 [†]
24:6n-3	0.17 ± 0.01	0.12 ± 0.00 [†]
22:6n-3	2.21 ± 0.05	2.26 ± 0.03 [‡]
Total n-3	4.02 ± 0.03	4.33 ± 0.02 ^{‡*}

Table 6-2. Fatty acid composition of cell phospholipids of HepG2 cells supplemented with 5 µg/ml 20:3n-6 alone or treated with the Δ 6 desaturase inhibitor, SC-26196.

HepG2 cells were cultured as described and supplemented with 5 µg/ml 20:3n-6 or with SC-26196 for 48 h. Values marked with an asterisk are significantly different from untreated cells as determined using either the Mann Whitney U test for nonparametric data (†) or the Student's T test for normally distributed data (‡) ($p < 0.05$).

6.3.2 Effect of SC-26196 on DPA metabolism

Treatment of DPA-supplemented HepG2 cells with SC-26196 caused an increase in the level of DPA in cell phospholipids, increasing from $3.49 \pm 0.31\%$ total fatty acids in untreated cells to $6.2 \pm 0.53\%$ total fatty acids in treated cells (Figure 6-5). The level of EPA increased marginally, although not significantly ($p > 0.05$), in treated HepG2 cells supplemented with DPA compared with untreated HepG2 cells (Table 6-3). The level of 24:5n-3, the elongation product of DPA, increased significantly ($p < 0.05$) from $1.06 \pm 0.32\%$ total fatty acids in untreated cells supplemented with DPA to $3.28 \pm 0.32\%$ total fatty acids in supplemented cells treated with SC-26196. The level of post $\Delta 6$ desaturase products, 24:6n-3 and DHA, in cell phospholipids was significantly reduced ($p < 0.05$) in treated cells compared with untreated cells. The level of DHA in phospholipids was reduced from $6.98 \pm 0.40\%$ total fatty acids in untreated cells supplemented with DPA, to $2.59 \pm 0.11\%$ total fatty acids in supplemented cells treated with SC-26196. These results are summarised in Table 6-3.

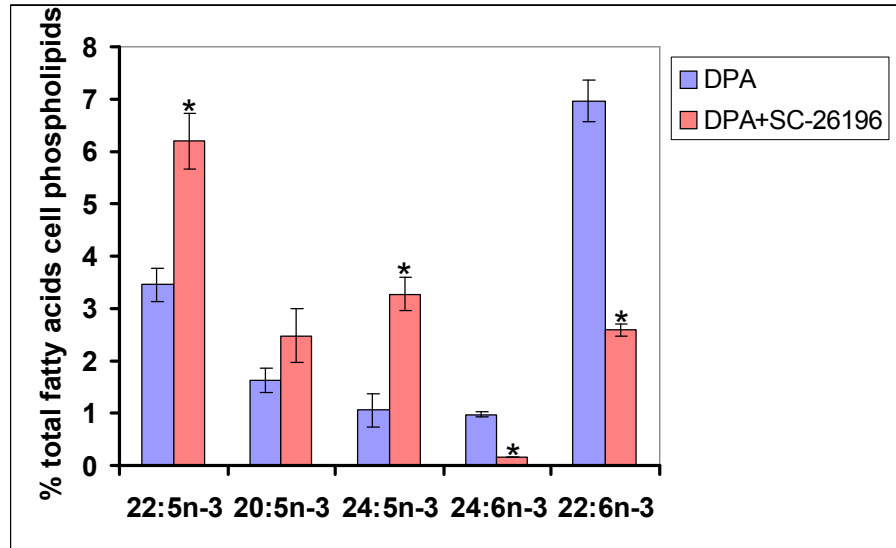


Figure 6-5. The accumulation of docosapentaenoic acid (22:5n-3, DPA) and its metabolites in HepG2 cells supplemented with 5 µg/ml DPA alone or treated with the Δ 6 desaturase inhibitor, SC-26196.

HepG2 cells were cultured as described and supplemented with 5 µg/ml DPA alone (blue) or with SC-26196 (pink) for 48 h.

* Indicates means are significantly different ($p < 0.05$) from untreated HepG2 cells supplemented with 5 µg/ml DPA.

	5 µg/ml DPA	5 µg/ml DPA+ 2 µM SC-26196
Fatty acid	% total fatty acids	
Total Saturates	33.51 ± 0.18	30.94 ± 0.26*
18:1n-9 OA	15.13 ± 0.17	15.99 ± 0.32
Total Monounsaturates	43.81 ± 0.67	45.82 ± 1.64
20:3n-9	1.67 ± 0.84	1.36 ± 0.66
Total n-9	20.22 ± 0.92	20.47 ± 0.86
18:2n-6 LA	0.87 ± 0.04	1.01 ± 0.11
18:3n-6	0.13 ± 0.01	0.14 ± 0.01
20:3n-6	0.51 ± 0.02	0.53 ± 0.03
20:4n-6 AA	2.66 ± 0.06	2.75 ± 0.12
22:5n-6 DPA n-6	0.16 ± 0.01	0.14 ± 0.004
Total n-6	4.61 ± 0.26	4.82 ± 0.36
18:3n-3 ALA	0.01 ± 0.01	0.02 ± 0.01
20:5n-3 EPA	1.63 ± 0.24	2.47 ± 0.52
22:5n-3 DPAn-3	3.49 ± 0.31	6.20 ± 0.53*
24:5n-3	1.06 ± 0.32	3.28 ± 0.32*
24:6n-3	0.97 ± 0.05	0.18 ± 0.004*
22:6n-3 DHA	6.98 ± 0.40	2.59 ± 0.11*
Total n-3	15.20 ± 1.02	15.91 ± 1.35

Table 6-3. Fatty acid composition of cell phospholipids of HepG2 cells supplemented with 5 µg/ml docosapentaenoic acid (22:5n-3, DPA) alone or treated with the Δ 6 desaturase inhibitor, SC-26196.

HepG2 cells were cultured as described and supplemented with 5 µg/ml DPA alone or with SC-26196 for 48 h. Values marked with an asterisk are significantly different from untreated cells as determined by the Independent Student t-test ($p < 0.05$).

6.4 DISCUSSION

The fatty acid composition of HepG2 cells following treatment with the specific D6D inhibitor, SC-26196, is consistent with the requirement of D6D in the conversion of ALA and DPA to n-3 LCPUFA in HepG2 cells. The reduced accumulation of D6D products in both ALA- and DPA- supplemented HepG2 cells, treated with SC-26196, is consistent with a large body of evidence indicating that the same D6D acts on ALA and 24:5n-3 (94;133). The reduced accumulation of 24:6n-3 from DPA, concomitant with a decrease in the level of DHA in cells treated with SC-26196 indicates that 24:6n-3 is an intermediate in the biosynthesis of DHA as described *in vitro* (94;95) and *in vivo* (211). Although SC-26196 has been used by others (209), this is the first study to demonstrate changes in membrane fatty acid composition, a net effect of conversion and incorporation, as a result of treatment with SC-26196 and demonstrates the integral role of D6D in influencing the fatty acid composition of cell membranes.

Treatment of DPA- supplemented cells with SC-26196 marginally increased the accumulation of EPA in cell phospholipids. However, the level of 24:5n-3 in cell phospholipids increased significantly with SC-26196-treatment, suggesting that DPA is preferentially elongated, towards the synthesis of DHA. That is, the retroconversion of DPA to EPA does not appear to limit the accumulation of DHA. The comparatively low accumulation of 24 carbon fatty acids in HepG2 cell phospholipids suggests that its preferred fate is further elongation, then desaturation and finally oxidation to yield DHA. This suggests that events post-D6D in the conversion of fatty acid may regulate the accumulation of DHA from n-3 fatty acid precursors but cannot exclude the importance of D6D in this process.

In conclusion, the use of SC-26196 clearly demonstrates the absolute requirement for D6D in the synthesis of DHA from DPA and provides further evidence for the use of the HepG2 cell line as a model of fatty acid desaturation and elongation. In addition, it was demonstrated that retroconversion of DPA to EPA may not be a major limitation in the synthesis of DHA from DPA. The non-linear accumulation of DHA from DPA suggests that other steps involved in fatty acid conversion regulate the accumulation of DHA.

7 Co-supplementation of HepG2 cells with DPA and potential competitive inhibitors of D6D; LA and DTA

7.1 INTRODUCTION

The involvement of numerous enzymes in the desaturation/elongation pathway suggests there are several steps that may regulate the conversion of substrate fatty acids to LCPUFA. In Chapter 4 it was demonstrated that the accumulation of DHA reached a plateau following supplementation with increasing concentrations of DPA. The dose-dependent accumulation of the D6D precursor, 24:5n-3 and its product, 24:6n-3, in phospholipids of cells supplemented with DPA suggests that the limitation was due to events following the $\Delta 6$ desaturation of 24:5n-3 rather than a limitation at D6D. These events include peroxisomal β -oxidation, translocation of DHA out of the peroxisome and acylation of DHA into membrane lipids. The availability of fatty acids in the diacylglycerol pool for phospholipid biosynthesis may also contribute to the accumulation of DHA from DPA.

Partial β -oxidation in the peroxisome is the final step in the synthesis of DHA and involves several enzymatic reactions. There are two complete sets of β -oxidation enzymes present in the peroxisome (99;212). Straight chain acyl-CoA oxidase is responsible for the initial oxidation of very long chain fatty acyl-CoAs. The enoyl-CoA esters of straight chain fatty acids are then hydrated and subsequently dehydrogenated by L-bifunctional protein. The last step of the β -oxidation process, the thiolitic cleavage, is performed by both sterol carrier protein X and the classic 3-ketoacyl-CoA thiolase (99) (see Chapter One, Figure 1-5, for more detail).

Consequently, saturation of the enzymes involved in peroxisomal β -oxidation may also affect the accumulation of DHA from n-3 fatty acid precursors.

This chapter aimed to examine accumulation of DHA and other n-3 PUFA in HepG2 cells co-supplemented with DPA and increasing amounts of LA. LA is a substrate for D6D and is converted to AA and 22:5n-6. Since LA is the preferred substrate over 24:5n-3 (95), it was hypothesised that supplementation of HepG2 cells with DPA and increasing amounts of LA would significantly reduce the accumulation of DHA. Insights into the role of D6D in affecting the accumulation of DHA from DPA was hoped to be gained by examining the fatty acid composition of HepG2 cells supplemented with DPA and increasing concentrations of LA.

DPA and DTA are substrates for peroxisomal β -oxidation (Figure 7-1). It was hypothesised that increasing concentrations of DTA would reduce the accumulation of DHA in cells supplemented with DPA by utilising enzymes involved in β -oxidation. DTA is also a substrate of elongation to form 24:4n-6, which then becomes a substrate for D6D. Therefore, competition between 24:4n-6 and 24:5n-3 for D6D may also exist, in which case, the level of 24:6n-3, the Δ 6 desaturation product of 24:5n-3, will decrease in cells supplemented with DPA with increasing concentrations of DTA (Figure 7-1). Insights into the role of peroxisomal β -oxidation in affecting the accumulation of DHA from DPA was hoped to be gained by examining the fatty acid composition of HepG2 cells supplemented with DPA and increasing concentrations of DTA.

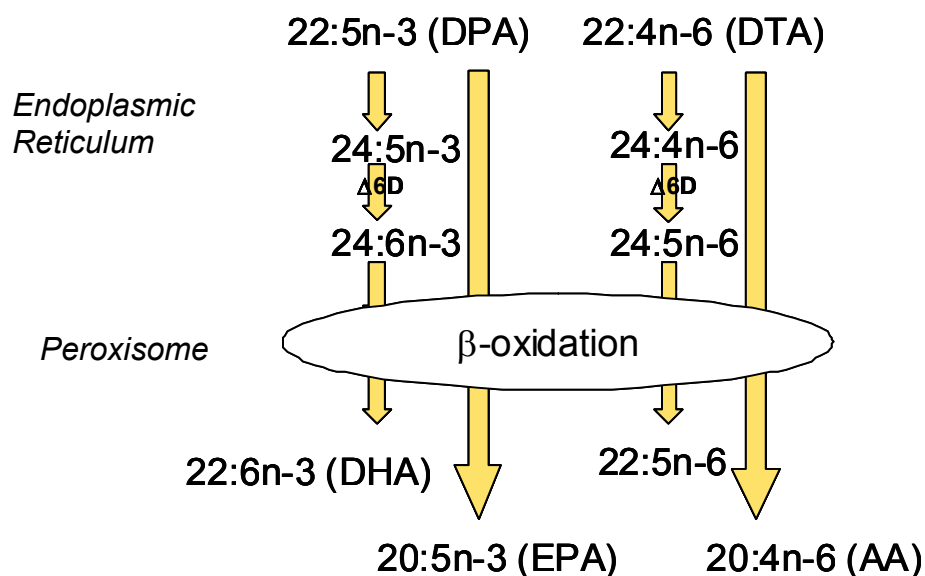


Figure 7-1. Entry of docosapentaenoic acid (22:5n-3, DPA) and docosatetraenoic acid (22:4n-6, DTA), or their respective metabolites, into the peroxisome to undergo partial β -oxidation.

7.2 MATERIALS AND METHODS

The materials and methods used were those described in Chapter 2, with the following changes.

7.2.1 Cell Culture

HepG2 cells were cultured and seeded into 6 well plates as described in Section 3.2.1. After 72 h, the medium was removed and replaced with serum-free DMEM supplemented with 5 $\mu\text{g/ml}$ DPA alone, or in addition to increasing amounts of LA (2.5-10 $\mu\text{g/ml}$) or DTA (2.5-10 $\mu\text{g/ml}$), bound to fatty acid-free BSA. The reciprocal experiment was also performed. In the reciprocal experiment, 72 h after seeding HepG2 cells, the medium was removed and replaced with serum-free DMEM supplemented with 5 $\mu\text{g/ml}$ DTA alone, or in addition to increasing amounts of DPA (2.5-10 $\mu\text{g/ml}$), bound to fatty acid-free BSA. Stock solutions of these fatty acids were prepared in ethanol at a concentration of 10 mg/ml and diluted in serum-free

medium to achieve the final desired concentration. The media was refreshed after 24 h with the appropriate concentration of fatty acid(s) bound to BSA. After 48 h incubation with the respective fatty acids, cells were harvested for fatty acid analysis (See section 2.2.7).

7.2.2 Fatty acid analysis

Total lipids were extracted from cells following procedures described in Chapter 2.

7.2.3 Statistical analysis

Statistical analyses were performed on at least three independent experiments as described in Chapter 2.

7.3 RESULTS

7.3.1 Co-supplementation of HepG2 cells with DPA and increasing concentrations of LA

The fatty acid composition of HepG2 cell phospholipids was examined following supplementation with DPA and increasing amounts of LA and was found to have no significant effect ($p > 0.05$) on the level of DHA in cell phospholipids (Figure 7-3). The level of EPA, the retroconversion product of DPA, in cell phospholipids was significantly reduced ($p < 0.05$) in LA co-supplemented HepG2 cells compared with control cells (DPA only) (Figure 7-3). There was a significant dose-dependent increase in the level of LA in HepG2 cell phospholipids following supplementation with DPA and LA (Figure 7-2). The level of AA in cell phospholipids was significantly increased ($p < 0.05$) in cells co-supplemented with DPA and 10 $\mu\text{g/ml}$ LA (

Table 7-1). The level of DPA in HepG2 cell phospholipids was unaffected ($p > 0.05$) by co-supplementation with LA (Figure 7-3). The level of monounsaturated fatty

acids in cell phospholipids was significantly decreased ($p < 0.05$), concurrent with a significant increase ($p < 0.05$) in the level of LA in cells supplemented with DPA and co-supplemented with LA. These results are summarised in Table 7-1.

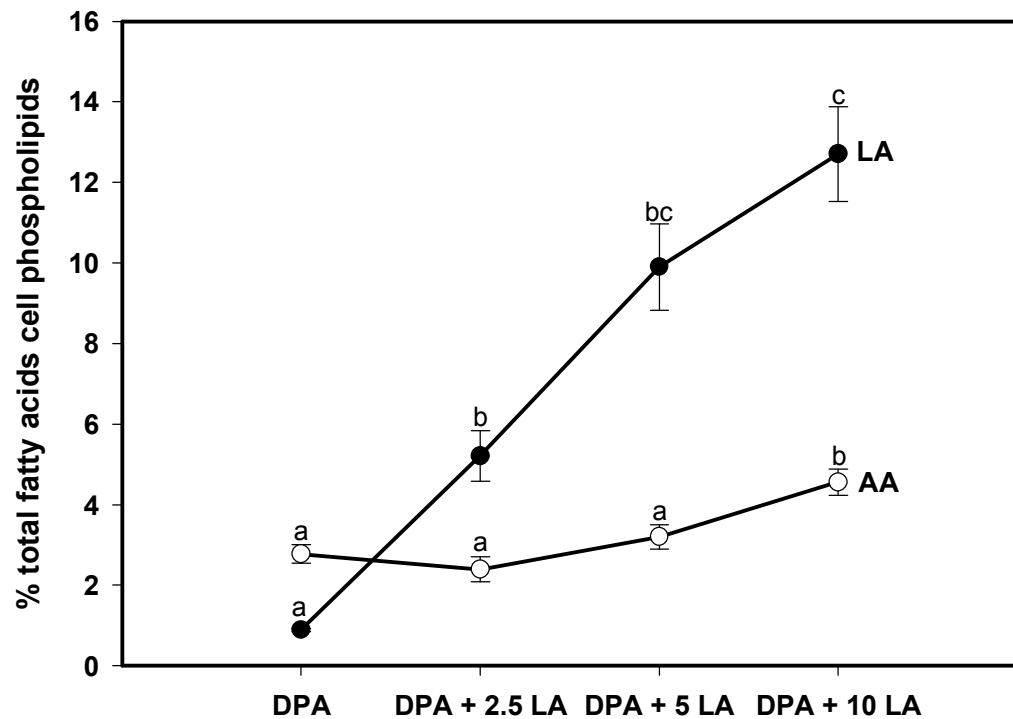


Figure 7-2. The accumulation of linoleic acid (18:2n-6, LA) and arachidonic acid (20:4n-6, AA) in HepG2 cells supplemented with 5 μ g/ml docosapentaenoic acid (22:5n-3, DPA) alone or with the addition of 2.5, 5 or 10 μ g/ml LA.

HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means \pm SE of three independent experiments with three replicates in each experiment. Values with different symbols are significantly different from each other ($p < 0.05$) for each fatty acid as determined by one-way ANOVA and Bonferroni post-hoc tests.

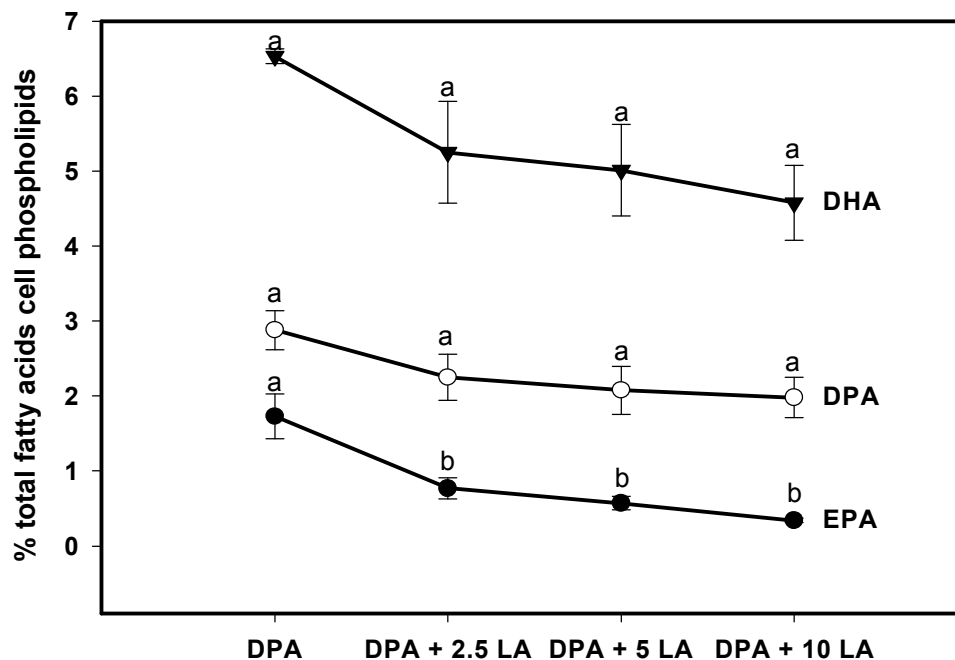


Figure 7-3. The accumulation of eicosapentaenoic acid (20:5n-3, EPA), docosapentaenoic acid (22:5n-3, DPA) and docosahexaenoic acid (22:6n-3, DHA) in HepG2 cells supplemented with 5 $\mu\text{g}/\text{ml}$ docosapentaenoic acid (22:5n-3, DPA) alone or with the addition of 2.5, 5 or 10 $\mu\text{g}/\text{ml}$ linoleic acid (18:3n-3, LA).

HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means \pm SE of three independent experiments with three replicates in each experiment. Values with different symbols are significantly different from each other ($p < 0.05$) for each fatty acid as determined by one-way ANOVA and Bonferroni post-hoc tests.

Fatty acid	% total fatty acids							
	5 µg/ml DPA*		5 µg/ml DPA + 2.5 µg/ml LA		5 µg/ml DPA + 5 µg/ml LA		5 µg/ml DPA + 10 µg/ml LA	
Total Saturates	33.76	± 0.61 ^a	33.42	± 0.81 ^a	33.74	± 1.07 ^a	34.79	± 0.88 ^a
18:1n-9 OA	14.25	± 0.38 ^a	13.90	± 0.62 ^a	12.31	± 0.78 ^{ab}	10.56	± 0.82 ^b
Total Monounsaturates	42.89	± 0.74 ^a	40.58	± 1.27 ^a	36.68	± 1.85 ^{ab}	31.70	± 1.97 ^b
20:3n-9	2.57	± 0.04 ^a	1.22	± 0.67 ^a	1.09	± 0.55 ^a	0.98	± 0.49 ^a
Total n-9	21.45	± 0.17 ^a	24.09	± 2.94 ^a	21.40	± 2.95 ^a	18.18	± 2.57 ^a
18:2n-6 LA	0.89	± 0.04 ^a	5.21	± 0.63 ^b	9.09	± 1.07 ^{bc}	12.70	± 1.18 ^c
18:3n-6	0.11	± 0.01 ^a	0.09	± 0.04 ^a	0.16	± 0.08 ^b	0.18	± 0.09 ^b
20:3n-6	0.46	± 0.02 ^a	0.56	± 0.23 ^a	0.90	± 0.39 ^a	1.26	± 0.57 ^a
20:4n-6 AA	2.77	± 0.23 ^a	2.39	± 0.31 ^a	3.02	± 0.30 ^a	4.56	± 0.33 ^b
22:5n-6	0.15	± 0.00 ^a	0.12	± 0.10 ^a	0.12	± 0.10 ^a	0.12	± 0.11 ^a
Total n-6	4.45	± 0.31 ^a	10.86	± 0.12 ^b	15.74	± 0.58 ^c	21.13	± 1.10 ^d
18:3n-3 ALA	0.20	± 0.19 ^a	0.11	± 0.05 ^a	0.11	± 0.06 ^a	0.12	± 0.06 ^a
20:5n-3 EPA	1.73	± 0.30 ^a	0.77	± 0.14 ^b	0.57	± 0.09 ^b	0.34	± 0.03 ^b
22:5n-3 DPA	2.88	± 0.26 ^a	2.25	± 0.31 ^a	2.08	± 0.32 ^a	1.98	± 0.27 ^a
24:5n-3	0.85	± 0.08 ^a	0.69	± 0.15 ^a	0.66	± 0.16 ^a	0.65	± 0.12 ^a
24:6n-3	0.64	± 0.02 ^a	0.73	± 0.06 ^a	0.54	± 0.11 ^a	0.54	± 0.03 ^a
22:6n-3 DHA	6.53	± 0.10 ^a	5.25	± 0.68 ^a	5.01	± 0.61 ^a	4.58	± 0.50 ^a
Total n-3	13.86	± 0.77 ^a	10.36	± 1.46 ^a	9.51	± 1.21 ^a	8.66	± 1.00 ^a

Table 7-1. Fatty acid composition of cell phospholipids of HepG2 cells supplemented with 5 µg/ml docosapentaenoic acid (DPA) alone or with the addition of 2.5, 5 or 10 µg/ml linoleic acid (18:2n-6, LA). HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means ± SE of three independent experiments with three replicates in each experiment. Values with different superscripts are significantly different from each other ($p < 0.05$) as determined by One-Way ANOVA with Bonferroni post-hoc test. *Experiments performed concurrent with those described in Chapter 4.

7.3.2 Co-supplementation of HepG2 cells with DPA and DTA

Co-supplementation of HepG2 cells with 5 µg/ml DPA and increasing concentrations of DTA significantly reduced ($p < 0.05$) the accumulation of DHA in cell phospholipids (Figure 7-4). The level of EPA, the retroconversion product of DPA, in cell phospholipids was significantly reduced ($p < 0.05$) in co-supplemented HepG2 cells, decreasing from $0.91 \pm 0.13\%$ total fatty acids in cells supplemented with 5 µg/ml DPA to $0.28 \pm 0.05\%$ total fatty acid in cells supplemented with 5 µg/ml DPA and 10 µg/ml DTA. The level of DTA and AA in cell phospholipids was dose-dependent, increasing with the addition of DPA to the media (Figure 7-4). The level of 24:4n-6 and 24:5n-6 in cell phospholipids was also linear, increasing with increasing concentrations of DTA in the media. The level of the elongation product of DPA, 24:5n-3 and the $\Delta 6$ desaturation product, 24:6n-3 was not significantly affected ($P > 0.05$) by DTA supplementation. These results are summarised in Table 7-2.

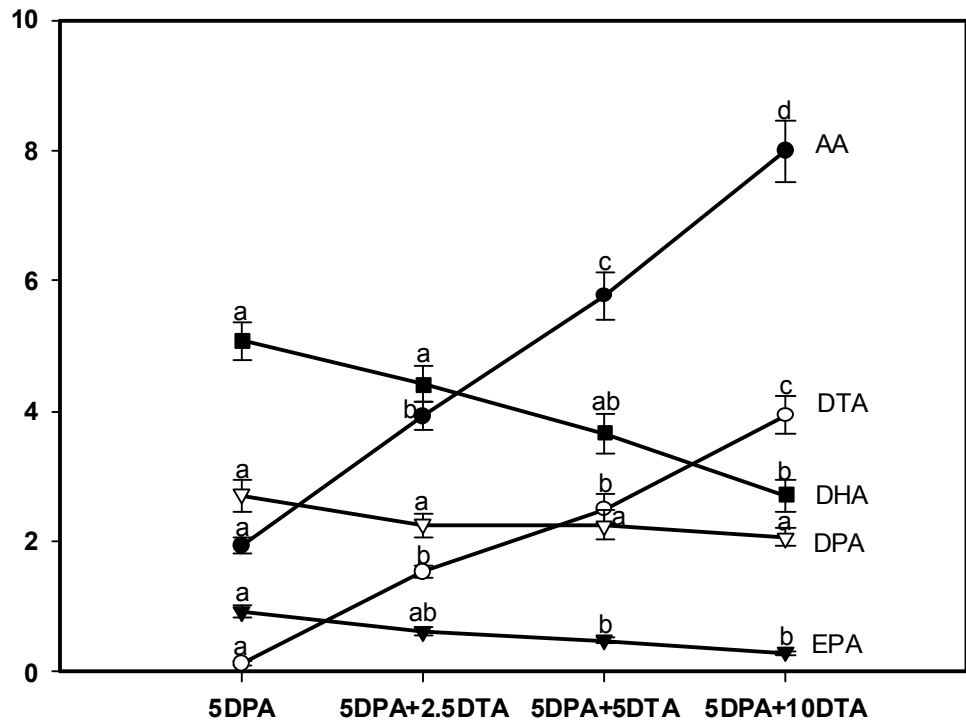


Figure 7-4. The accumulation of docosapentaenoic acid (22:5n-3, DPA) and docosatetraenoic acid (22:4n-6, DTA) and the β -oxidation products, eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, AA), in HepG2 cells supplemented with 5 μ g/ml DPA or with the addition of 2.5, 5 or 10 μ g/ml DTA.

HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means \pm SE of three independent experiments with three replicates in each experiment.

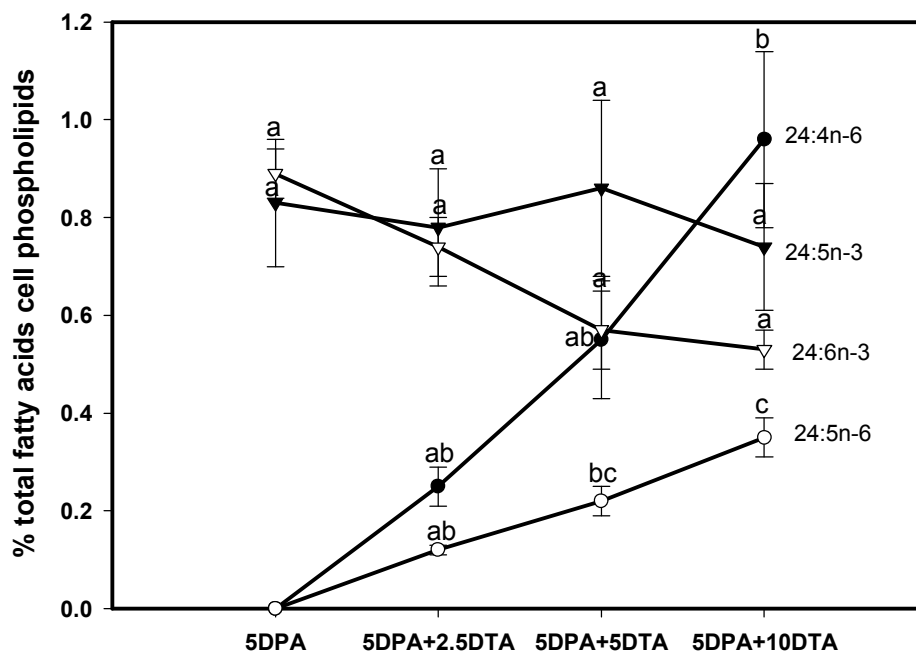


Figure 7-5. The accumulation of 24:5n-3, 24:6n-3, 24:4n-6 and 24:5n-6 in HepG2 cells supplemented with 5 μ g/ml docosapentaenoic acid (22:5n-3, DPA) or with the addition of 2.5, 5 or 10 μ g/ml docosatetraenoic acid (22:4n-6, DTA).

HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means \pm SE of three independent experiments with three replicates in each experiment.

Fatty acid	% total fatty acids							
	5 µg/ml DPA		5 µg/ml DPA + 2.5 µg/ml DTA		5 µg/ml DPA + 5 µg/ml DTA		5 µg/ml DPA + 10 µg/ml DTA	
Total Saturates	32.51	± 0.72 ^a	34.23	± 0.47 ^a	34.99	± 0.67 ^a	36.05	± 1.15 ^a
18:1n-9 OA	15.59	± 0.45 ^a	14.74	± 0.56 ^{ab}	13.63	± 0.56 ^{ab}	12.31	± 0.52 ^b
Total Monounsaturates	45.70	± 0.56 ^a	42.12	± 0.26 ^a	39.98	± 0.76 ^b	36.70	± 1.20 ^b
20:3n-9	1.19	± 0.68 ^a	1.11	± 0.61 ^a	1.04	± 0.62 ^a	0.92	± 0.54 ^a
Total n-9	27.38	± 2.53 ^a	25.22	± 2.35 ^a	23.73	± 2.39 ^a	21.35	± 2.32 ^a
18:2n-6 LA	0.77	± 0.06 ^a	0.78	± 0.06 ^a	0.81	± 0.11 ^a	0.90	± 0.16 ^a
18:3n-6	0.10	± 0.02 ^a	0.10	± 0.003 ^a	0.09	± 0.012 ^a	0.09	± 0.012 ^a
20:3n-6	0.18	± 0.09 ^a	0.23	± 0.13 ^a	0.25	± 0.15 ^a	0.27	± 0.15 ^a
20:4n-6 AA	1.82	± 0.10 ^a	3.75	± 0.19 ^b	5.50	± 0.33 ^c	7.69	± 0.52 ^d
22:4n-6 DTA	0.11	± 0.04 ^a	1.50	± 0.11 ^b	2.50	± 0.31 ^b	4.06	± 0.40 ^c
22:5n-6	0.34	± 0.02 ^a	0.21	± 0.09 ^a	0.31	± 0.02 ^a	0.29	± 0.08 ^a
24:4n-6	nd		0.25	± 0.05 ^{ab}	0.56	± 0.16 ^{ab}	0.99	± 0.25 ^b
24:5n-6	nd		0.12	± 0.02 ^{ab}	0.23	± 0.03 ^{bc}	0.37	± 0.04 ^c
Total n-6	5.55	± 1.16 ^a	9.36	± 0.78 ^{ab}	12.20	± 0.72 ^{bc}	16.47	± 0.89 ^c
18:3n-3 ALA	0.15	± 0.005 ^a	0.17	± 0.04 ^a	0.17	± 0.03 ^a	0.17	± 0.03 ^a
20:5n-3 EPA	0.91	± 0.13 ^a	0.61	± 0.08 ^{ab}	0.46	± 0.06 ^b	0.28	± 0.05 ^b
22:5n-3 DPA	2.65	± 0.34 ^a	2.21	± 0.27 ^a	2.23	± 0.32 ^a	2.05	± 0.20 ^a
24:5n-3	0.83	± 0.18 ^a	0.79	± 0.16 ^a	0.89	± 0.25 ^a	0.75	± 0.18 ^a
24:6n-3	0.88	± 0.07 ^a	0.73	± 0.09 ^a	0.55	± 0.12 ^a	0.54	± 0.06 ^a
22:6n-3 DHA	4.87	± 0.27 ^a	4.23	± 0.32 ^a	3.54	± 0.39 ^{ab}	2.55	± 0.27 ^b
Total n-3	10.83	± 1.18 ^a	9.21	± 1.00 ^a	8.28	± 1.04 ^a	6.71	± 0.70 ^a

Table 7-2. Fatty acid composition of cell phospholipids of HepG2 cells supplemented with 5 µg/ml docosapentaenoic acid (22:5n-3, DPA) or with the addition of 2.5, 5 and 10 µg/ml docosatetraenoic acid (22:4n-6, DTA). HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means ± SE of three independent experiments with three replicates in each experiment. Values with different superscripts are significantly different from each other ($p < 0.05$) as determined by One-Way ANOVA with Bonferroni post-hoc test. ND; not detected.

7.3.2 continued Co-supplementation of HepG2 cells with DPA and DTA

The reciprocal experiment, in which cells were supplemented with 5 µg/ml DTA and increasing concentrations of DPA, was also performed. There was a linear and significant increase in the level of DHA in HepG2 cell phospholipids in cells supplemented with DTA and increasing concentrations of DPA (Figure 7-6). The level of DPA, and its retroconversion product, EPA, also increased dose-dependently. Surprisingly, the level of AA, the retroconversion product of DTA, increased significantly with the addition of DPA, although was not dependent on the dose of DPA (Figure 7-6). The elongation product of DPA, 24:5n-3 and its subsequent Δ 6 desaturation product, 24:6n-3, increased dose-dependently in HepG2 cell phospholipids (Figure 7-7). The level of 24:4n-6 and 24:5n-6 in cell phospholipids was significantly reduced in HepG2 cells supplemented with 5 µg/ml DPA and increasing concentrations of DPA. The total level of saturated fatty acids in HepG2 cell phospholipids was significantly increased following supplementation with 5 µg/ml DTA and increasing concentrations of DPA. The total level of monounsaturated fatty acids in cell phospholipids was significantly decreased ($p < 0.05$) in HepG2 cells supplemented with 5 µg/ml DTA and increasing concentrations of DPA. These results are summarised in

Table 7-3.

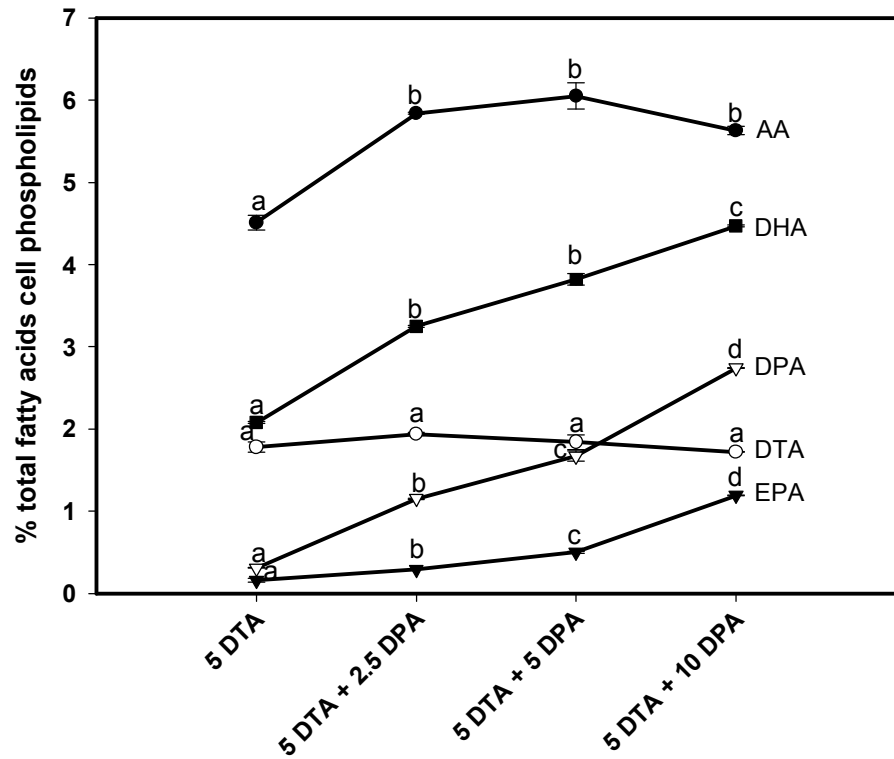


Figure 7-6. The accumulation of docosatetraenoic acid (22:4n-6, DTA) and docosapentaenoic acid (22:5n-3, DPA) and the β -oxidation products, arachidonic acid (20:4n-6, AA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) in HepG2 cells supplemented with 5 μ g/ml DTA or with the addition of 2.5, 5 or 10 μ g/ml DPA.

HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means \pm SE of three independent experiments with three replicates in each experiment.

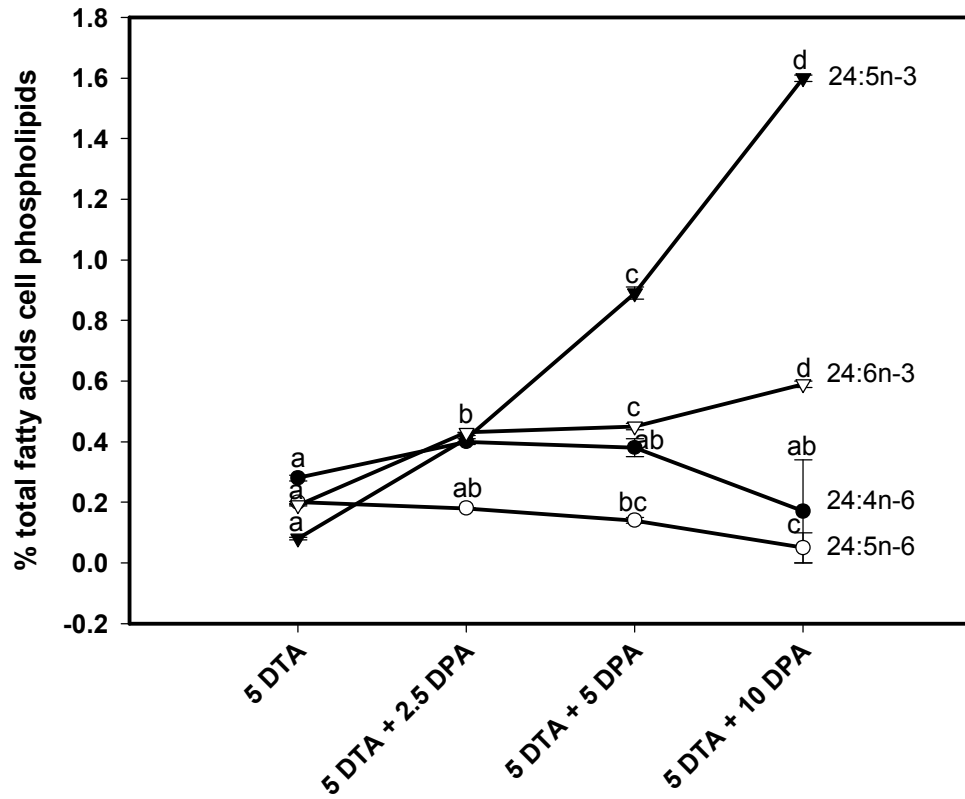


Figure 7-7. The accumulation of 24:4n-6, 24:5n-6 and 24:5n-3, 24:6n-3 in HepG2 cells supplemented with 5 $\mu\text{g/ml}$ docosatetraenoic acid (22:4n-6, DTA) or with the addition of 2.5, 5 or 10 $\mu\text{g/ml}$ docosapentaenoic acid (22:5n-3, DPA).

HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means \pm SE of three independent experiments with three replicates in each experiment.

Fatty acid	5 µg/ml DTA	5 µg/ml DTA + 2.5 µg/ml DPA	5 µg/ml DTA + 5 µg/ml DPA	5 µg/ml DTA + 10 µg/ml DPA
	% total fatty acids cell phospholipids			
Total Saturates	34.75 ± 0.01 ^a	36.21 ± 0.06 ^b	36.81 ± 0.07 ^c	37.48 ± 0.10 ^d
18:1n-9 OA	16.44 ± 0.24 ^a	15.34 ± 0.01 ^b	14.32 ± 0.05 ^{bc}	13.38 ± 0.21 ^c
Total Monounsaturates	44.38 ± 0.67 ^a	41.12 ± 0.13 ^{ab}	38.46 ± 0.35 ^{bc}	36.32 ± 0.58 ^c
20:3n-9	2.71 ± 0.02 ^a	2.20 ± 0.01 ^b	2.04 ± 0.03 ^b	1.88 ± 0.04 ^c
Total n-9	23.97 ± 0.29 ^a	21.63 ± 0.05 ^b	20.14 ± 0.20 ^{bc}	18.79 ± 0.32 ^c
18:2n-6 LA	1.16 ± 0.17 ^a	0.92 ± 0.01 ^a	1.13 ± 0.23 ^a	1.07 ± 0.17 ^a
18:3n-6	0.10 ± 0.002 ^a	0.10 ± 0.000 ^a	0.11 ± 0.002 ^a	0.11 ± 0.002 ^a
20:3n-6	0.49 ± 0.01 ^a	0.59 ± 0.003 ^b	0.60 ± 0.01 ^b	0.61 ± 0.001 ^b
20:4n-6 AA	4.51 ± 0.09 ^a	5.84 ± 0.01 ^b	6.05 ± 0.16 ^b	5.63 ± 0.05 ^b
22:4n-6 DTA	1.78 ± 0.06 ^a	1.94 ± 0.02 ^a	1.84 ± 0.09 ^a	1.72 ± 0.003 ^a
22:5n-6	2.25 ± 0.05 ^a	1.73 ± 0.01 ^b	1.26 ± 0.06 ^c	0.80 ± 0.01 ^d
24:4n-6	0.28 ± 0.01 ^a	0.40 ± 0.01 ^b	0.38 ± 0.03 ^{ab}	0.34 ± 0.01 ^{ab}
24:5n-6	0.20 ± 0.003 ^a	0.18 ± 0.001 ^{ab}	0.14 ± 0.01 ^{bc}	0.11 ± 0.007 ^c
Total n-6	11.15 ± 0.14 ^a	12.01 ± 0.19 ^b	12.05 ± 0.07 ^b	10.64 ± 0.04 ^a
18:3n-3 ALA			0.01 ± 0.002	0.01 ± 0.001
20:5n-3 EPA	0.16 ± 0.02 ^a	0.29 ± 0.001 ^b	0.50 ± 0.01 ^c	1.19 ± 0.004 ^d
22:5n-3 DPA	0.31 ± 0.004 ^a	1.15 ± 0.01 ^b	1.67 ± 0.06 ^c	2.74 ± 0.003 ^d
24:5n-3	0.08 ± 0.004 ^a	0.41 ± 0.01 ^b	0.89 ± 0.02 ^c	1.60 ± 0.01 ^d
24:6n-3	0.19 ± 0.003 ^a	0.43 ± 0.002 ^b	0.45 ± 0.01 ^c	0.59 ± 0.01 ^d
22:6n-3 DHA	2.08 ± 0.01 ^a	3.25 ± 0.01 ^b	3.82 ± 0.07 ^b	4.47 ± 0.01 ^c
Total n-3	4.15 ± 0.23 ^a	6.50 ± 0.02 ^b	8.50 ± 0.08 ^c	11.58 ± 0.13 ^d

Table 7-3. Fatty acid composition of cell phospholipids of HepG2 cells supplemented with 5 µg/ml docosatetraenoic acid (22:4n-6, DTA) or with the addition of 2.5, 5 and 10 µg/ml docosapentaenoic acid (22:5n-3, DPA). HepG2 cells were cultured as described and supplemented with fatty acids for 48 h. Values are means ± SE of three independent experiments with three replicates in each experiment. Values with different superscripts are significantly different from each other ($p < 0.05$) as determined by One-Way ANOVA with Bonferroni post-hoc test.

7.4 DISCUSSION

This chapter examined the fatty acid composition of HepG2 cell phospholipids following co-supplementation with DPA and LA or DTA, respectively. The lack of effect of increasing concentrations of LA on the accumulation of DHA from DPA suggests that competition between LA and 24:5n-3 for D6D may not be a key regulator in the conversion of n-3 fatty acid precursors to DHA. Furthermore, the level of 24:6n-3, the immediate precursor of DHA, was not reduced following co-supplementation with LA despite a greater affinity of D6D for LA over 24:5n-3 (95) suggesting that other processes may limit the accumulation of DHA from DPA such as peroxisomal β -oxidation or acylation of fatty acids into cell phospholipids.

In contrast, there was a significant reduction in the accumulation of DHA from DPA with increasing concentrations of DTA, the n-6 homologue of DPA. Co-supplementation of HepG2 cells with 10 $\mu\text{g/ml}$ DTA and 5 $\mu\text{g/ml}$ DPA reduced the level of DHA in cell phospholipids by almost 50% compared with a 30% reduction in cells co-supplemented with 10 $\mu\text{g/ml}$ LA. That is, the effect of LA on the conversion of DPA to DHA appears to be weaker compared to DTA. This may reflect the preferential incorporation of LA into cell phospholipids compared with DTA as demonstrated by the slower rates of esterification of 22-carbon PUFA compared with 18-carbon PUFA (96). Alternatively, the reported changes in the fatty acid composition of cell phospholipids following co-supplementation may be indicative of fatty acid partitioning into triglyceride pools. There is evidence to suggest that AA and EPA compete for esterification into cellular phospholipids and that excess cellular fatty acids enter cellular lipid storage pools, predominantly triglycerides *in vitro* (213).

In cells supplemented with a fixed dose of DTA and increasing concentrations of DPA, the accumulation of AA, the retroconversion product of DTA, was surprisingly elevated. This may indicate that the peroxisomal β -oxidation pathway has a preference for n-6 fatty acids over n-3 fatty acids, specifically DTA over DPA or the preferential acylation of fatty acids into cell lipids. The preferential β -oxidation of n-6 fatty acids over n-3 fatty acids has been demonstrated in human skin fibroblasts (214;215). However, in isolated rat sertoli cells, the peroxisomal β -oxidation of labeled 24:5n-6 and 24:6n-3 did not show a difference between the n-6 and n-3 families (216). The substrate specificity of peroxisomal β -oxidation has been described in terms of substrate chain length with fatty acids with more than 20 carbons long being substrates for this pathway, regardless of fatty acid class (85;99;217). The increased accumulation of AA in DTA-supplemented cells with increasing concentrations of DPA may be indicative of the preferred elongation of DPA to 24:5n-3 which shunts DTA into the peroxisome. The utilisation of the elongation and Δ 6 desaturation enzymes by n-3 fatty acids may shunt DTA towards β -oxidation and spare the oxidation of n-3 fatty acids.

The changes in the fatty acid composition of HepG2 cells co-supplemented with DPA and DTA highlights peroxisomal β -oxidation as a possible regulator in the synthesis of DHA from fatty acid precursors. Partial peroxisomal β -oxidation is the final step in the synthesis of DHA from n-3 fatty acid precursors (85;94;178). The reduction in the accumulation of DHA in cells co-supplemented with DPA and DTA compared with DPA and LA indicates that peroxisomal β -oxidation may play a key regulatory role in the accumulation of DHA from DPA in addition to D6D. The significant reduction in the accumulation of DHA from DPA when DTA was present suggests that competition for enzymes involved in peroxisomal β -oxidation may

affect the synthesis of DHA from n-3 fatty acid precursors. Additionally, DHA is a substrate for peroxisomal β -oxidation itself that may limit its accumulation in cell membranes.

The importance of peroxisomal β -oxidation in the synthesis of DHA is underscored by the reduced levels of DHA in patients with peroxisomal biogenesis disorders such as Zellweger's syndrome and other genetic diseases affecting peroxisomal β -oxidation (99). The involvement of peroxisomal β -oxidation enzymes in the synthesis of DHA is exemplified by the ability of PPAR agonists to induce the expression of these enzymes (206;218) and increase the level of DHA in tissues *in vivo* (219;220). In this chapter, the fatty acid composition of HepG2 cell phospholipids was measured as a surrogate marker of peroxisomal β -oxidation. Further experiments which use labeled fatty acids and measure the concentration of fatty acid intermediates and labeled CO₂ would provide a direct measurement of β -oxidation and may help in validating the model used here. Future research should be directed towards understanding the regulatory role peroxisomal β -oxidation plays in the synthesis of DHA from n-3 fatty acid precursors.

8 General discussion

This thesis reported on the use of an *in vitro* cell system to examine the accumulation of fatty acids following supplementation with individual fatty acids in order to identify possible regulatory steps in their conversion and subsequent accumulation into cell phospholipids. Although the experiments presented in this thesis do not specifically address the enzymatic reactions involved in the desaturation/elongation pathway, they generate a number of interesting results. This approach was applied in an attempt to model the accumulation of fatty acids in cell lipids that may occur in humans following dietary fatty acid intervention, and, in particular, the accumulation of DHA from n-3 fatty acid precursors. Interpretation of human and animal dietary intervention studies is often complicated by the presence of numerous dietary fatty acids that compete for common enzymes in the desaturation/elongation pathway and affect fatty acid conversion. Here, I have described changes in the fatty acid composition of cell phospholipids in response to supplementation with a single fatty acid in isolation from other competing fatty acid substrates. The effect of n-6 fatty acids on the accumulation of n-3 fatty acids in HepG2 cell phospholipids was also examined in co-supplementation experiments.

The experiments described herein highlight the exchange of fatty acids as constituents of phospholipids and the capacity for the cell membrane to incorporate particular fatty acids. However, by measuring the fatty acid composition of cell lipids following fatty acid supplementation, the combined effect of several pathways, including fatty acid conversion and phospholipid biosynthesis, may affect the measured outcome. Yet these same pathways affect the accumulation of fatty acids into cell lipids in humans following dietary fatty acid supplementation and suggest that the cell system used here may provide a suitable model of fatty acid conversion

and accumulation *in vivo*. β -oxidation, carbon recycling and the metabolic state (including hormonal influences) will also affect the fatty acid composition of tissues *in vivo* and represents a major difference from *in vitro* fatty acid metabolism but does permit the investigation of the conversion and accumulation of fatty acids into cell lipids in isolation of such confounding influences as mentioned above.

In Chapter 3, the congruency between the accumulation of n-3 and n-6 fatty acids in cell lipids *in vitro* with that described *in vivo* was demonstrated and supports the use of this HepG2 cell model. ALA-supplementation in both animals and humans has consistently shown a direct relationship between the level of EPA in tissues and plasma and the dietary intake of ALA. However, ALA-supplementation in animals and humans has little or no effect on the level of DHA in tissues (6;7;136;139;141). Similarly, in HepG2 cell phospholipids, there was a non-linear relationship between the accumulation of DHA and the concentration of ALA in the media. In the absence of competing fatty acid substrates, a limitation in the accumulation of DHA from ALA in HepG2 cell phospholipids was observed. The use of D6D twice in the conversion of ALA to DHA implies that the competition between ALA and 24:5n-3 may limit the accumulation of DHA in cell phospholipids. By identifying and measuring the 24 carbon n-3 fatty acids, 24:5n-3 and 24:6n-3, further insights into the possible steps involved in regulating the accumulation of DHA from ALA were gained.

This thesis is one of the first studies to report the accumulation of 24:5n-3 and 24:6n-3 in cell lipids. The linear accumulation of 24:5n-3 in cell phospholipids and the curvilinear accumulation of 24:6n-3, which paralleled the accumulation of DHA, suggests that competition between ALA and 24:5n-3 for D6D may be involved in

regulating the accumulation of DHA from ALA *in vitro*. The pattern of accumulation of n-3 fatty acids in HepG2 cell phospholipids following supplementation with ALA suggests that, even in the absence of competing substrates, ALA may be primarily involved in regulating the conversion of ALA to DHA and may not be a suitable supplement for elevating the level of DHA in tissues *in vivo*.

Competition between ALA and 24:5n-3 for D6D may provide one explanation for the poor accumulation of DHA following ALA-supplementation *in vivo*. In addition, the preferential metabolic fate of ALA *in vivo* will also affect the availability of ALA in its conversion to DHA. ALA is a substrate for β -oxidation in humans and is partitioned to this catabolic pathway more readily than palmitic, stearic and oleic acid (221). The proportion of ingested labelled ALA used in β -oxidation can be estimated by measuring the appearance of labelled CO₂ on breath. Reports estimate that 15-33% of ingested ALA undergoes mitochondrial β -oxidation (31;32;151;222). Burdge and Calder (2005) (223) suggest that these values represent an approximate 30% underestimate of the actual amount of ALA that is β -oxidised due to trapping of ¹³CO₂ in bicarbonate pools (224) and indicates that ingested ALA is used extensively in β -oxidation. One possible explanation for the preferred use of ALA for β -oxidation is the greater affinity of carnitine:palmitoyl transferase-1, the rate limiting enzyme in mitochondrial fatty acid β -oxidation, for ALA compared to other unsaturated fatty acids such as OA, and LA (225). In addition to conversion to CO₂, carbon in acetyl-CoA generated by fatty acid β -oxidation may also be recycled and used in *de novo* fatty acid synthesis. The extent of partitioning of ALA towards β -oxidation and carbon recycling may be important in regulating the availability of

ALA for conversion to DHA and provides another explanation for the poor accumulation of DHA from ALA observed *in vivo*.

In Chapter 4, EPA and DPA were identified as more efficient n-3 fatty acid precursors in elevating the content of DHA in HepG2 cell phospholipids compared with ALA. However, *in vivo*, the level of DHA in plasma lipids was unaffected following supplementation with pure EPA (153;186-189), suggesting a greater level of regulation. While the efficacy of EPA in elevating the level of DHA in humans has been examined previously (153;186-189), the potential role of DPA in elevating n-3 LCPUFA status has been largely ignored despite recent research which shows that DPA contributes almost 30% total n-3 LCPUFA in our diet (2). Traditionally, fish oils have been used to increase the n-3 LCPUFA status of individuals, either by supplementing the diet with fish oil capsules or consuming foods enriched with microencapsulated fish oil, both of which rely heavily on a declining global fish supply. Meat, poultry and game products contribute almost 50% of our intake of DPA (5) and may provide an alternative. The enrichment of foods with DPA by supplementing livestock feeds with plant sources of ALA (226) may be an alternative to increase the n-3 LCPUFA status of the population, particularly as Australians consume greater quantities of meat or poultry compared with fish or seafood (2). Future studies should be directed towards examining the accumulation of DHA from increased dietary DPA

Direct supplementation of preformed DHA is currently the most effective way to increase the content of DHA in human tissues (154;186;188;189). In a cross-study meta-regression analysis, Arterburn *et al.* (2006) (179) demonstrated that the level of DHA in plasma phospholipids increased in a dose-dependent, saturable manner in

response to dietary DHA, as was also suggested by Vidgren *et al.* (1997) (227). Similarly, in this thesis, the accumulation of DHA in HepG2 cell phospholipids following supplementation with increasing concentrations of preformed DHA, reached a plateau and indicates the correlation between the accumulation of DHA *in vitro* with that *in vivo*.

Peroxisomal β -oxidation may also play a regulatory role in the accumulation of DHA in cell membranes from n-3 fatty acids. The accumulation of DHA in cell phospholipids reached a plateau in cells supplemented with increasing concentration of EPA or DPA. The linear accumulation of the D6D substrate, 24:5n-3, and the D6D product, 24:6n-3, suggests that the curvilinear accumulation of DHA in cells supplemented with EPA or DPA may not be due to saturation of D6D but rather another regulatory step in the conversion of EPA and DPA to DHA. Co-supplementation of HepG2 cells with DPA and DTA suggests a role for peroxisomal β -oxidation in regulating the accumulation of DHA from n-3 fatty acids. The accumulation of DHA in cells supplemented with DPA, and the accumulation of AA in cells supplemented with DTA, involves peroxisomal β -oxidation. There was a significant reduction in the accumulation of DHA from DPA when HepG2 cells were supplemented with increasing concentrations of DTA, the n-6 equivalent of DPA. Furthermore, the level of EPA was significantly reduced with the addition of increasing concentrations of DTA, concomitant with an increase in the level of AA and suggests that competition between DPA and DTA for enzymes involved in peroxisomal β -oxidation may limit the accumulation of DHA.

The potential for peroxisomal β -oxidation to influence the accumulation of LCPUFA, such as DHA, in tissues has been demonstrated in animals treated with

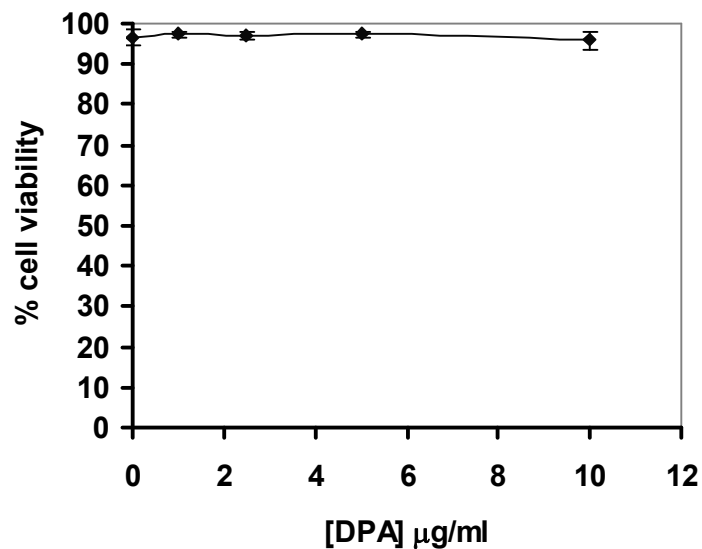
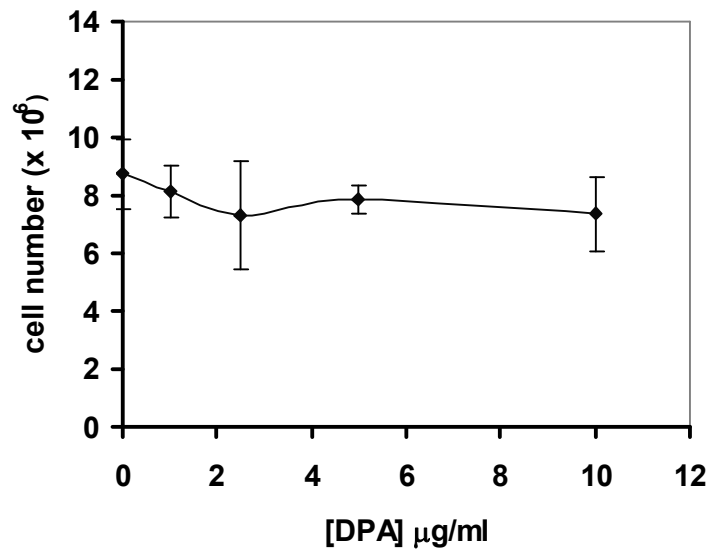
PPAR agonists, such as fibrates (114;219;220). For example, treatment of rats with clofibrate caused an almost 2-fold increase in the level of DHA in cardiomyocytes compared with untreated rats (219). The development of PPAR α -null mice also indicates the role of PPAR in activating enzymes of peroxisomal β -oxidation in the synthesis of DHA. In PPAR α -null mice, fed diets containing soybean oil (54% LA and 8% ALA total fatty acids), the level of DHA that accumulated in liver phospholipids was significantly reduced compared with the wild-type (220). However, extrapolation of fatty acid metabolism between animals and humans in regards to the involvement of peroxisomal β -oxidation must be with caution due to differences in the relative expression of PPAR between species (228;229). The human liver reportedly expresses PPAR at levels that are approximately an order of magnitude lower than rat liver (230).

The human HepG2 cell line may be a useful tool in further investigations into the regulatory role of peroxisomal β -oxidation, however will always be limited by its transformed phenotype. The protein and mRNA expression of PPAR in HepG2 cells is reportedly lower than that of normal human hepatocytes (230;231). Therefore, such studies should consider transfecting HepG2 cells with a PPAR expression vector. This would allow a thorough examination of the effects of synthetic (fibrates) and natural PPAR agonists (which include polyunsaturated, monounsaturated and saturated fatty acids (117;232)) on the conversion of substrate fatty acids and the accumulation of their long chain metabolites in cell lipids, particularly DHA.

Both D6D and peroxisomal β -oxidation are important regulatory steps governing the accumulation of DHA from n-3 fatty acid precursors and appear to have a concerted

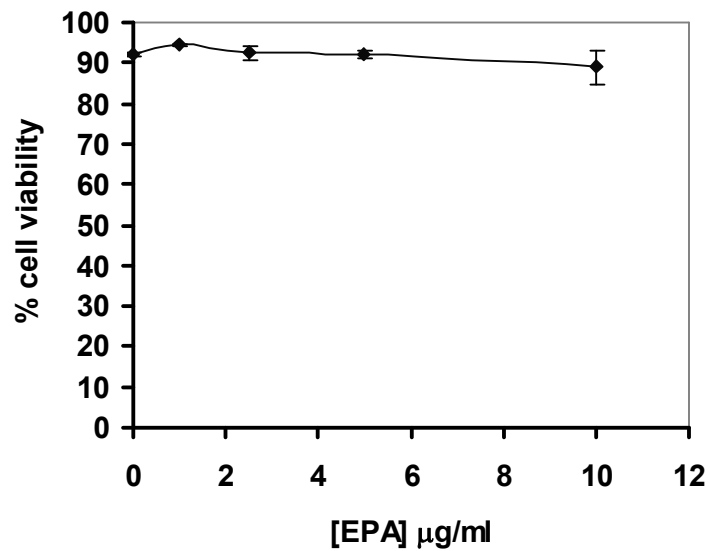
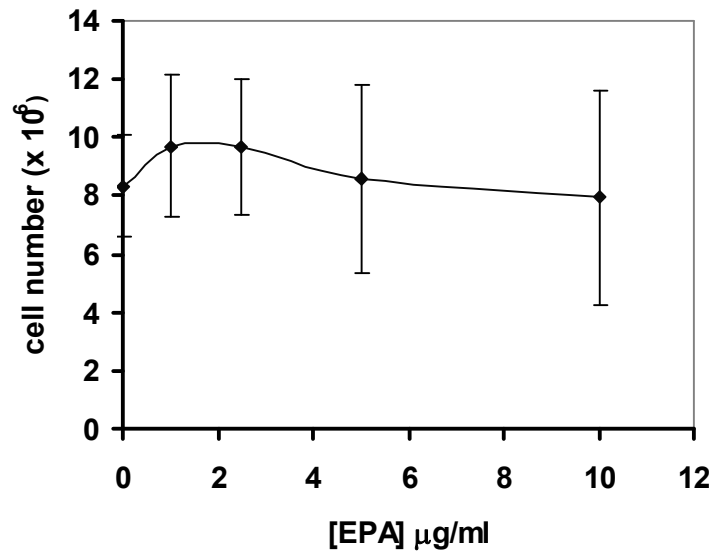
role in modulating the fatty acid composition of cell membranes. *In vitro* models are advantageous in their ability to examine the accumulation of fatty acids from a single fatty acid supplement. Clearly, the activity of enzymes involved in fatty acid conversion, as well as those enzymes involved in lipid biosynthesis, will be important determinants of membrane fatty acid composition, in addition to the intake/supplementation of fatty acids. This thesis presents a strong case for the use of HepG2 cells in future experiments aimed at examining potential regulatory steps in the conversion of fatty acids. The research presented in this thesis suggests that a greater understanding of the regulation of fatty acid synthesis may unlock mechanisms to elevate the level of n-3 LCPUFA in tissues without the need for direct supplementation. Ultimately, a greater understanding of fatty acid synthesis will help in defining dietary approaches that will increase the n-3 LCPUFA status of animals and humans to provide more sustainable sources of n-3 LCPUFA.

Appendix 1: Cell number and viability (Chapter 4)



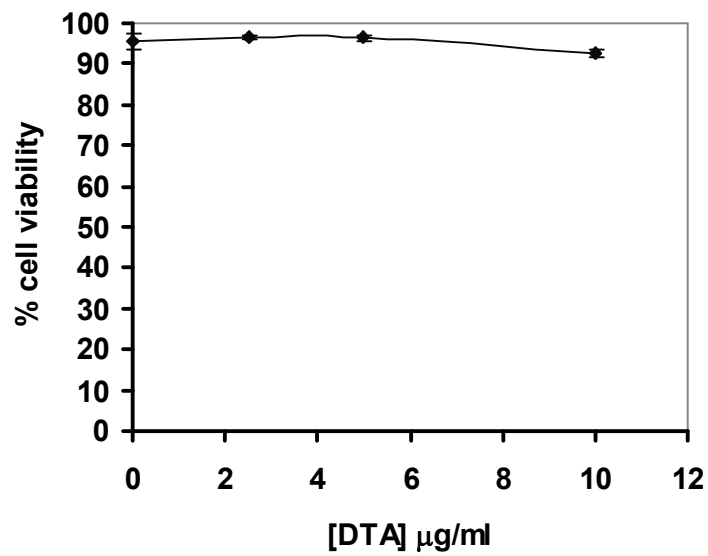
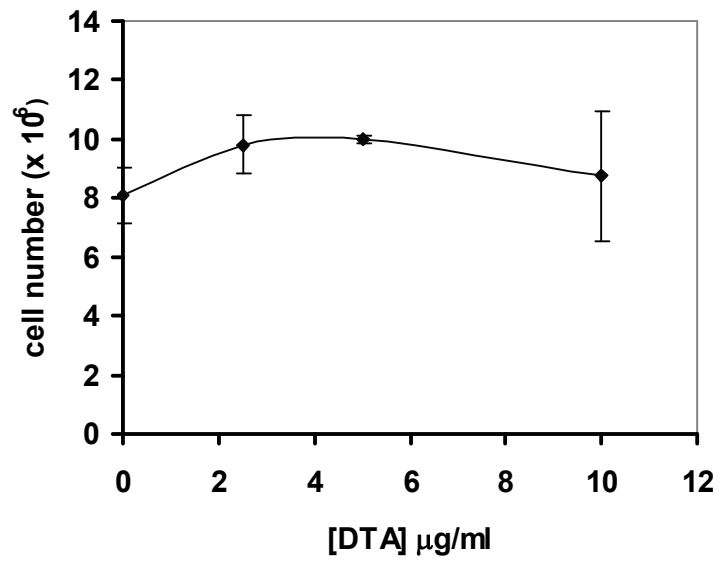
Appendix Figure 1-1. Cell number and viability of HepG2 cells supplemented with increasing concentrations of docosapentaenoic acid (22:5n-3, DPA).

HepG2 cells were incubated for 48 h with increasing concentrations of DPA bound to BSA in serum-free DMEM. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.



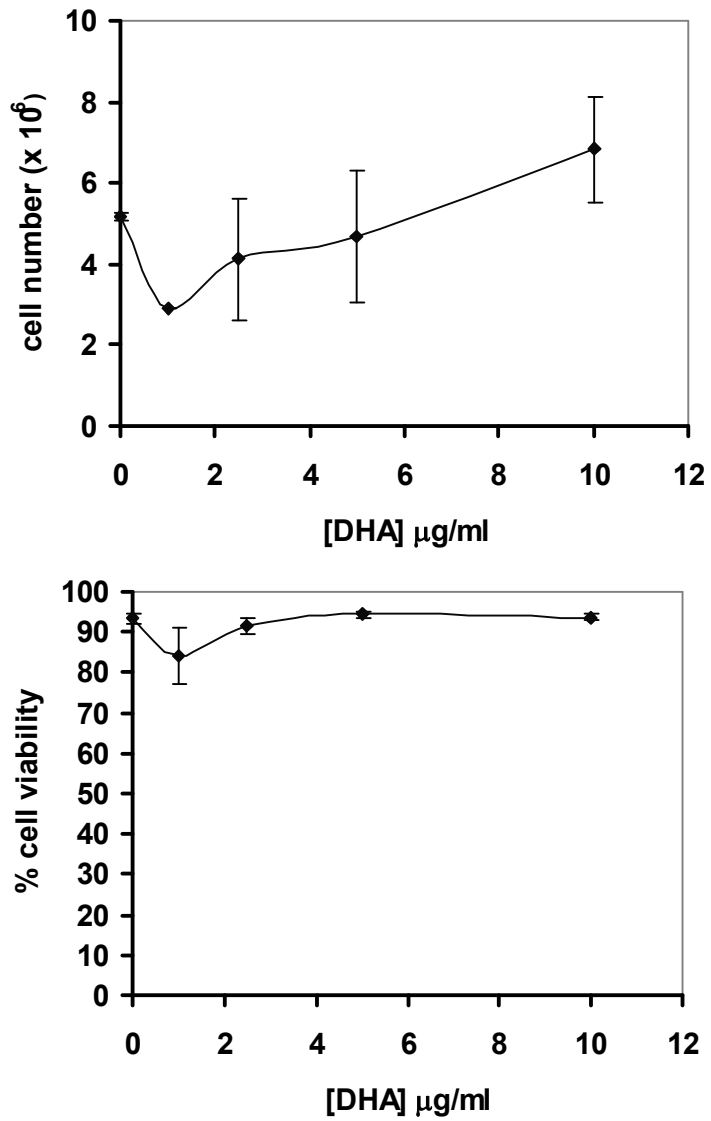
Appendix Figure 1-2. Cell number and viability of HepG2 cells supplemented with increasing concentrations of eicosapentaenoic acid (20:5n-3, EPA).

HepG2 cells were incubated for 48 h with increasing concentrations of EPA bound to BSA in serum-free DMEM. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.



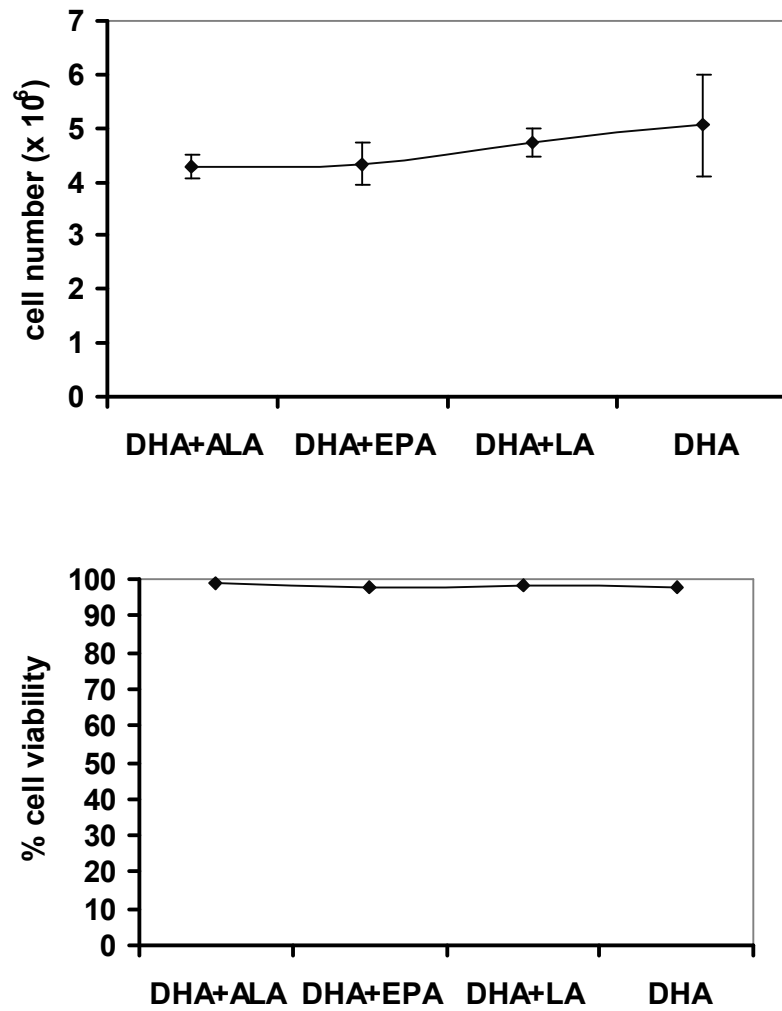
Appendix Figure 1-3. Cell number and viability of HepG2 cells supplemented with increasing concentrations of docosatetraenoic acid (22:4n-6, DTA).

HepG2 cells were incubated for 48 h with increasing concentrations of DTA bound to BSA in serum-free DMEM. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.



Appendix Figure 1-4. Cell number and viability of HepG2 cells supplemented with increasing concentrations of docosahexaenoic acid (22:6n-3, DHA).

HepG2 cells were incubated for 48 h with increasing concentrations of DHA bound to BSA in serum-free DMEM. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.



Appendix Figure 1-5. Cell number and viability of HepG2 cells supplemented with docosahexaenoic acid (22:6n-3, DHA) alone, or in addition to α -linolenic acid (18:3n-3, ALA), linoleic acid (18:2n-6, LA) or eicosapentaenoic acid (20:5n-3, EPA).

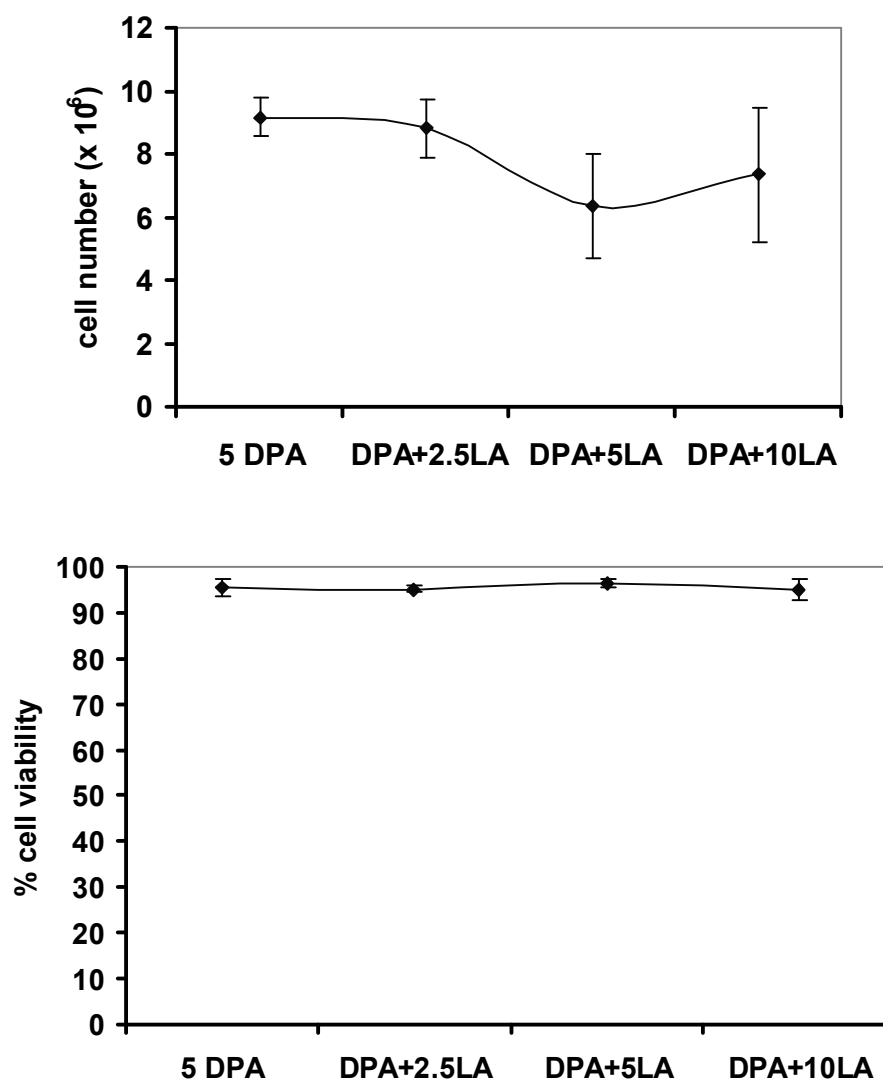
HepG2 cells were supplemented with fatty acids for 2 h. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.

Appendix 2: Fatty acid composition of HepG2 cells treated with SC-26196 (Chapter 6)

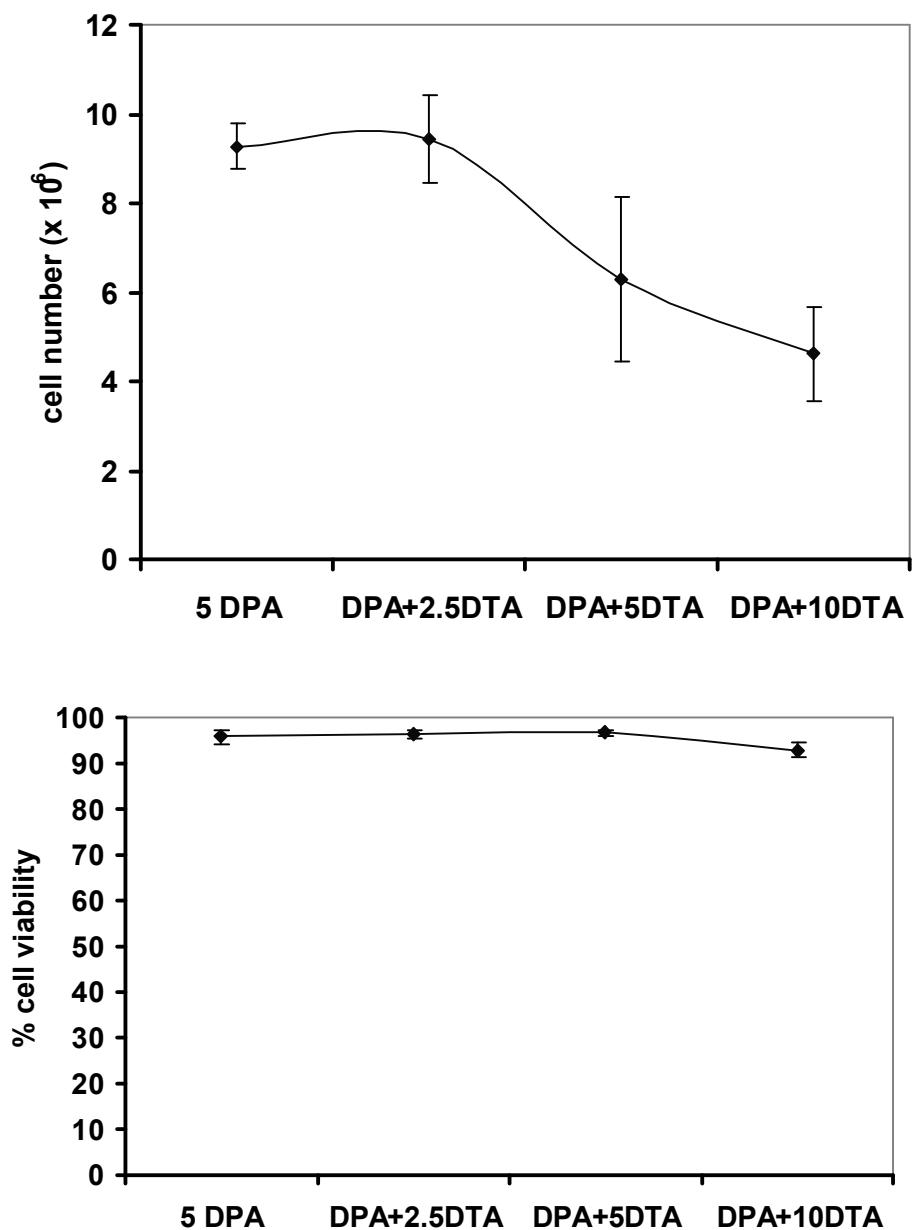
Fatty acid	Serum free		Serum free + 2 μ M SC-26196	
	% total fatty acids			
Total Saturates	28.67	\pm 1.64	27.67	\pm 1.47
18:1n-9 OA	18.23	\pm 0.89	19.60	\pm 1.20
Total Monounsaturates	55.76	\pm 2.46	58.33	\pm 1.62
20:3n-9	3.30	\pm 1.63	1.52	\pm 0.74
Total n-9	26.16	\pm 1.22	25.28	\pm 1.12
18:2n-6 LA	0.89	\pm 0.07	0.93	\pm 0.05
18:3n-6	0.13	\pm 0.03	0.12	\pm 0.01
20:3n-6	0.51	\pm 0.03	0.51	\pm 0.02
20:4n-6 AA	3.02	\pm 0.09	3.00	\pm 0.13
22:5n-6 DPAn-6	0.22	\pm 0.02	0.20	\pm 0.03
Total n-6	5.00	\pm 0.31	5.25	\pm 0.50
18:3n-3 ALA			0.05	\pm 0.05
20:3n-3	0.04	\pm 0.003	0.03	\pm 0.03
20:5n-3 EPA	0.12	\pm 0.03	0.15	\pm 0.02
22:5n-3 DPA	0.37	\pm 0.04	0.41	\pm 0.05
24:5n-3	0.11	\pm 0.04	0.16	\pm 0.06
24:6n-3	0.22	\pm 0.03	0.16	\pm 0.06
22:6n-3 DHA	2.62	\pm 0.12	2.61	\pm 0.16
Total n-3	5.19	\pm 0.27	5.32	\pm 0.27

Appendix Figure 2-1. Fatty acid composition of cell phospholipids of HepG2 cells maintained in serum-free medium or treated with the Δ 6 desaturase inhibitor, SC-26196. Values are means \pm SE.

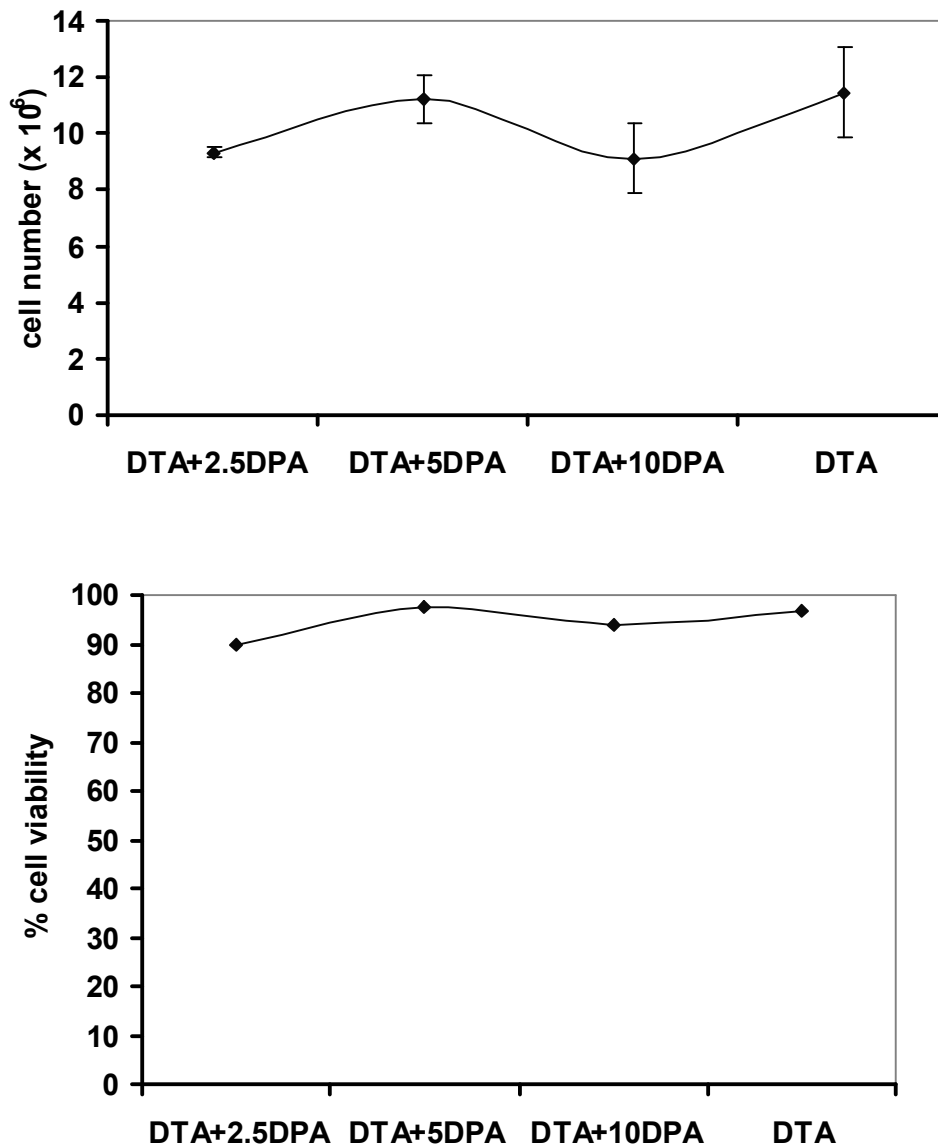
Appendix 3: Cell number and viability (Chapter 7)



Appendix Figure 3-1. Cell number and viability of HepG2 cells supplemented with 5 $\mu\text{g/ml}$ docosapentaenoic acid (22:5n-3, DPA) alone or with the addition of 2.5, 5 or 10 $\mu\text{g/ml}$ linoleic acid (18:2n-6, LA). HepG2 cells were supplemented with fatty acids for 48 h. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.



Appendix Figure 3-2. Cell number and viability of HepG2 cells supplemented with 5 $\mu\text{g/ml}$ docosapentaenoic acid (22:5n-3, DPA) alone or with the addition of 2.5, 5 or 10 $\mu\text{g/ml}$ docosatetraenoic acid (22:4n-6, DTA). HepG2 cells were supplemented with fatty acids for 48 h. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.



Appendix Figure 3-3. Cell number and viability of HepG2 cells supplemented with 5 $\mu\text{g/ml}$ docosatetraenoic acid (22:4n-6, DTA) alone or with the addition of 2.5, 5 or 10 $\mu\text{g/ml}$ docosapentaenoic acid (22:5n-3, DPA). HepG2 cells were supplemented with fatty acids for 48 h. Cell number was determined at the end of the supplementation period as described in Section 2.2.4. Cell viability was determined by trypan blue exclusion as described in Section 2.2.5.

BIBLIOGRAPHY

1. Lichtenstein AH, Appel LJ, Brands M et al. Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. *Circulation* 2006;114:82-96.
2. Howe P, Meyer B, Record S, Baghurst K. Dietary intake of long-chain omega-3 polyunsaturated fatty acids: contribution of meat sources. *Nutrition* 2006;22:47-53.
3. McLennan W, Podger A. National Nutrition Survey Australia 1995. Canberra: Australian Government Publishing Service, 1998.
4. Hunter JE. n-3 fatty acids from vegetable oils. *Am J Clin Nutr.* 1990;51:809-14.
5. Meyer BJ, Mann NJ, Lewis JL, Milligan GC, Sinclair AJ, Howe PR. Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids* 2003;38:391-8.
6. Sanders TAB, Roshanai F. The influence of different types of omega-3 polyunsaturated fatty acids on blood lipids and platelet function in healthy volunteers. *Clin.Sci.* 1983;64:91-9.
7. Mantzioris E, James MJ, Gibson RA, Cleland LG. Dietary substitution with an α -linolenic acid-rich vegetable oil increases eicosapentaenoic acid concentrations in tissues. *Am.J.Clin.Nutr.* 1994;59:1304-9.
8. Simopoulos AP. Commentary on the workshop statement. Essentiality of and recommended dietary intakes for Omega-6 and Omega-3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 2000;63:123-4.
9. Parks EJ. Effect of dietary carbohydrate on triglyceride metabolism in humans. *J Nutr* 2001;131:2772S-4S.
10. Mantzioris E, Cleland LG, Gibson RA, Neumann MA, Demasi M, James MJ. Biochemical effects of a diet containing foods enriched with n-3 fatty acids. *Am J Clin Nutr.* 2000;72:42-8.
11. Hornstra G. Regulation of Prostanoid Production by Dietary Fatty Acids. *Med.Biol.* 1984;62:261-2.
12. Kinsella JE. Lipids, membrane receptors, and enzymes: Effects of dietary fatty acids. *JPEN* 1990;14:200-17.

13. Jump DB, Clarke SD, Thelen A, Liimatta M, Ren B, Badin MV. Dietary fat, genes, and human health. *Adv.Exp.Med.Biol.* 1997;422:167-76.
14. Gurr MI, Harwood JL, Frayn KN. *Lipid Biochemistry An Introduction* 5th Edition. Oxford, UK: Blackwell Science Ltd, 2002.
15. Burr GO, Burr MM. A new deficiency disease produced by the rigid exclusion of fat from the diet. *J Biol Chem* 1929;82:345-67.
16. Sinclair AJ, Attar-Bashi NM, Li D. What is the role of alpha-linolenic acid for mammals? *Lipids* 2002;37:1113-23.
17. Ayerza, R and Coates, W. CHIA seeds: new source of OMEGA-3 fatty acids, natural antioxidants, and dietetic fibre. 31-7-2001.
18. Calder PC. Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. *Brazilian Journal of Medical & Biological Research* 1998;31:467-90.
19. Meyer BJ, Howe PRC Lewis J. Food sources and intakes of omega-6 and omega-3 polyunsaturated fatty acids in Australia. 2001. IUNS, Vienna.
20. Sirtori CR, Galli C, Franceschini G. Fraudulent (and non fraudulent) fatty acids for human health. *Eur.J.Clin.Invest.* 1993;23:686-9.
21. James MJ, Gibson RA, Cleland LG. Dietary polyunsaturated fatty acids and inflammatory mediator production. *Am J Clin Nutr.* 2000;71:343S-8S.
22. Verbeke W, Sioen I, Pieniak Z, Van Camp J, De Henauw S. Consumer perception versus scientific evidence about health benefits and safety risks from fish consumption. *Public Health Nutr.* 2005;8:422-9.
23. Shepherd R. Influences on food choice and dietary behavior. *Forum Nutr.* 2005;36-43.
24. Domergue F, Abbadi A, Heinz E. Relief for fish stocks: oceanic fatty acids in transgenic oilseeds. *Trends Plant Sci.* 2005;10:112-6.
25. Ratledge C. Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. *Biochimie* 2004;86:807-15.
26. Sijtsma L, de Swaaf ME. Biotechnological production and applications of the omega-3 polyunsaturated fatty acid docosahexaenoic acid. *Appl.Microbiol.Biotechnol.* 2004;64:146-53.
27. Napier JA, Sayanova O, Qi B, Lazarus CM. Progress toward the production of long-chain polyunsaturated fatty acids in transgenic plants. *Lipids* 2004;39:1067-75.
28. Robert SS, Singh SP, Zhou XR et al. Metabolic engineering of Arabidopsis to produce nutritionally important DHA in seed oil. *Functional Plant Biology* 2005;32:1-7.

29. Curtis KR, McCluskey J.J., Wahl TI. Consumer Acceptance of Genetically Modified Food Products in the Developing World. *AgBioForum* 2004;7:70-5.
30. Emken EA, Adlof RO, Rakoff H, Rohwedder WK, Gulley RM. Human metabolic studies with deuterated alpha-linolenic acid. *Nutrition* 1992;8:213-4.
31. Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *Br.J.Nutr.* 2002;88:411-20.
32. Burdge GC, Jones AE, Wootton SA. Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*. *Br.J.Nutr.* 2002;88:355-64.
33. Hu FB, Stampfer MJ, Manson JE et al. Dietary intake of alpha-linolenic acid and risk of fatal ischemic heart disease among women. *Am.J.Clin.Nutr.* 1999;69:890-7.
34. Albert CM, Campos H, Stampfer MJ et al. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. *N.Engl.J Med.* 2002;346:1113-8.
35. de Lorgeril M, Renaud S, Mamelle N et al. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet* 1994;343:1454-9.
36. GISSI-Prevenzione Investigators (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *Lancet* 1999;354:447-55.
37. Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* 2002;106:2747-57.
38. Makrides M, Neumann M, Simmer K, Pater J, Gibson RA. Are long-chain polyunsaturated fatty acids essential nutrients in infancy? *Lancet* 1995;345:1463-8.
39. Agostoni C, Trojan S, Bellù R, Riva E, Giovannini M. Neurodevelopmental quotient of healthy term infants at 4 months and feeding practice: The role of long-chain polyunsaturated fatty acids. *Pediatr.Res.* 1995;38:262-6.
40. Carlson SE, Ford AJ, Werkman SH, Peeples JM, Koo WWK. Visual acuity and fatty acid status of term infants fed human milk and formulas with and without docosahexaenoate and arachidonate from egg yolk lecithin. *Pediatr.Res.* 1996;39:882-8.
41. Willatts P, Forsyth JS, DiModugno MK, Varma S, Colvin M. Influence of long-chain polyunsaturated fatty acids on infant cognitive function. *Lipids* 1998;33:973-80.
42. Birch EE, Garfield S, Hoffman DR, Uauy R, Birch DG. A randomized controlled trial of early dietary supply of long-chain polyunsaturated fatty

- acids and mental development in term infants. *Dev.Med.Child Neurol.* 2000;42:174-81.
43. Jensen CL, Voigt RG, Prager TC et al. Effects of maternal docosahexaenoic acid intake on visual function and neurodevelopment in breastfed term infants. *Am.J Clin.Nutr.* 2005;82:125-32.
 44. Cleland LG, Proudman SM, Hall C et al. A biomarker of n-3 compliance in patients taking fish oil for rheumatoid arthritis. *Lipids* 2003;38:419-24.
 45. Stene LC, Joner G. Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study. *Am.J.Clin.Nutr.* 2003;78:1128-34.
 46. Steyn NP, Mann J, Bennett PH et al. Diet, nutrition and the prevention of type 2 diabetes. *Public Health Nutr.* 2004;7:147-65.
 47. Rivellese AA, Maffettone A, Iovine C et al. Long-term effects of fish oil on insulin resistance and plasma lipoproteins in NIDDM patients with hypertriglyceridemia. *Diabetes Care* 1996;19:1207-13.
 48. Calder PC, Davis J, Yaqoob P, Pala H, Thies F, Newsholme EA. Dietary fish oil suppresses human colon tumour growth in athymic mice. *Clin.Sci.(Lond)* 1998;94:303-11.
 49. Chang WL, Chapkin RS, Lupton JR. Fish oil blocks azoxymethane-induced rat colon tumorigenesis by increasing cell differentiation and apoptosis rather than decreasing cell proliferation. *J Nutr.* 1998;128:491-7.
 50. Maclean CH, Newberry SJ, Mojica WA et al. Effects of omega-3 fatty acids on cancer risk: a systematic review. *JAMA* 2006;295:403-15.
 51. Bang HO, Dyerberg J, Sinclair HM. The composition of the Eskimo food in north western Greenland. *Am J Clin Nutr.* 1980;33:2657-61.
 52. Burr ML, Fehily AM, Gilbert JF et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet* 1989;2:757-61.
 53. Bucher HC, Hengstler P, Schindler C, Meier G. N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. *Am.J Med.* 2002;112:298-304.
 54. Hooper L, Thompson R, Harrison R et al. Omega 3 fatty acids for prevention and treatment of cardiovascular disease. *Cochrane.Database.Syst.Rev.* 2004;CD003177.
 55. He K, Song Y. Risks and benefits of omega 3 fats: a few thoughts on systematic review. *Br.Med.J.* 2006;332:915-6.
 56. Brunner E. Oily fish and omega 3 fat supplements. *Br.Med.J.* 2006;332:739-40.

57. Geleijnse JM, Brouwer IA, Feskens EJ. Risks and benefits of omega 3 fats: health benefits of omega 3 fats are in doubt. *Br.Med.J.* 2006;332:915-6.
58. Ness AR, Hughes J, Elwood PC, Whitley E, Smith GD, Burr ML. The long-term effect of dietary advice in men with coronary disease: follow-up of the Diet and Reinfarction trial (DART). *Eur.J Clin.Nutr.* 2002;56:512-8.
59. Kang JX, Leaf A. Prevention of fatal cardiac arrhythmias by polyunsaturated fatty acids. *Am J Clin Nutr* 2000;71:202S-7S.
60. McCann JC, Ames BN. Is docosahexaenoic acid, an n-3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals. *Am.J.Clin.Nutr.* 2005;82:281-95.
61. Willatts P, Forsyth JS. The role of long-chain polyunsaturated fatty acids in infant cognitive development. *Prostaglandins Leukot.Essent.Fatty Acids* 2000;63:95-100.
62. Makrides M, Neumann MA, Simmer K, Gibson RA. A critical appraisal of the role of dietary long-chain polyunsaturated fatty acids on neural indices of term infants: A randomized, controlled trial. *Pediatrics* 2000;105:32-8.
63. Simmer K, Patole S. Longchain polyunsaturated fatty acid supplementation in preterm infants. *Cochrane.Database.Syst.Rev.* 2004;CD000375.
64. Gibson RA, Chen W, Makrides M. Randomized trials with polyunsaturated fatty acid interventions in preterm and term infants: functional and clinical outcomes. *Lipids* 2001;36:873-83.
65. ISSFAL. RECOMMENDATIONS FOR INTAKE OF POLYUNSATURATED FATTY ACIDS IN HEALTHY ADULTS. 1-22. 2004.
66. Mathews CK, van Holde KE. *Biochemistry.* Redwood City, CA: Benjamin/Cummings, 1990.
67. Jump DB, Clarke SD. Regulation of gene expression by dietary fat. *Annu Rev Nutr* 1999;19:63-90.
68. Hamilton JA, Kamp F. How are free fatty acids transported in membranes? Is it by proteins or by free diffusion through the lipids? *Diabetes* 1999;48:2255-69.
69. Van Nieuwenhoven FA, van der Vusse GJ, Glatz JF. Membrane-associated and cytoplasmic fatty acid-binding proteins. *Lipids* 1996;31 Suppl:S223-S227.
70. Abumrad N, Coburn C, Ibrahimi A. Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. *Biochim.Biophys.Acta* 1999;1441:4-13.
71. Glatz JF, van der Vusse GJ. Cellular fatty acid-binding proteins: their function and physiological significance. *Prog.Lipid Res.* 1996;35:243-82.

72. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 1998;93:241-52.
73. Issemann I, Prince R, Tugwood J, Green S. A role for fatty acids and liver fatty acid binding protein in peroxisome proliferation? *Biochem.Soc.Trans.* 1992;20:824-7.
74. Frohnert BI, Hui TY, Bernlohr DA. Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. *J Biol.Chem.* 1999;274:3970-7.
75. Kerner J, Hoppel C. Fatty acid import into mitochondria. *Biochim.Biophys.Acta* 2000;1486:1-17.
76. Storch J, Thumser AE. The fatty acid transport function of fatty acid-binding proteins. *Biochim.Biophys.Acta* 2000;1486:28-44.
77. Ek BA, Cistola DP, Hamilton JA, Kaduce TL, Spector AA. Fatty acid binding proteins reduce 15-lipoxygenase-induced oxygenation of linoleic acid and arachidonic acid. *Biochim.Biophys.Acta* 1997;1346:75-85.
78. Murphy EJ, Edmondson RD, Russell DH, Colles S, Schroeder F. Isolation and characterization of two distinct forms of liver fatty acid binding protein from the rat. *Biochim.Biophys.Acta* 1999;1436:413-25.
79. Jump DB. Fatty acid regulation of gene transcription. *Crit Rev.Clin.Lab Sci.* 2004;41:41-78.
80. Nakamura MT, Cho HP, Clarke SD. Regulation of hepatic delta-6 desaturase expression and its role in the polyunsaturated fatty acid inhibition of fatty acid synthase gene expression in mice. *J Nutr* 2000;130:1561-5.
81. Ide T, Kobayashi H, Ashakumary L et al. Comparative effects of perilla and fish oils on the activity and gene expression of fatty acid oxidation enzymes in rat liver. *Biochim.Biophys.Acta* 2000;1485:23-35.
82. McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur.J Biochem.* 1997;244:1-14.
83. Clarke SD, Gasperikova D, Nelson C, Lapillonne A, Heird WC. Fatty acid regulation of gene expression: a genomic explanation for the benefits of the mediterranean diet. *Ann N.Y.Acad.Sci.* 2002;967:283-98.
84. Wilson MD, Blake WL, Salati LM, Clarke SD. Potency of polyunsaturated and saturated fats as short-term inhibitors of hepatic lipogenesis in rats. *J.Nutr.* 1990;120:544-52.
85. Su HM, Moser AB, Moser HW, Watkins PA. Peroxisomal straight-chain Acyl-CoA oxidase and D-bifunctional protein are essential for the retroconversion step in docosahexaenoic acid synthesis. *J Biol.Chem.* 2001;276:38115-20.

86. Cinti DL, Cook L, Nagi MN, Suneja SK. The fatty acid chain elongation system of mammalian endoplasmic reticulum. *Prog.Lipid Res.* 1992;31:1-51.
87. Sprecher HW, Baykousheva SP, Luthria DL, Mohammed BS. Differences in the regulation of biosynthesis of 20- versus 22- carbon polyunsaturated fatty acids. *Prostaglandins Leukot.Essent.Fatty Acids* 1995;52:99-101.
88. Nakamura MT, Nara TY. Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu.Rev.Nutr.* 2004;24:345-76.
89. Sprecher H. Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochim.Biophys.Acta* 2000;1486:219-31.
90. Suneja SK, Osei P, Cook L, Nagi MN, Cinti DL. Enzyme site-specific changes in hepatic microsomal fatty acid chain elongation in streptozotocin-induced diabetic rats. *Biochim.Biophys.Acta* 1990;1042:81-5.
91. Guillou H, D'andrea S, Rioux V et al. Distinct roles of endoplasmic reticulum cytochrome b5 and fused cytochrome b5-like domain for rat Delta6-desaturase activity. *J.Lipid Res.* 2004;45:32-40.
92. Williard DE, Nwankwo JO, Kaduce TL et al. Identification of a fatty acid delta6-desaturase deficiency in human skin fibroblasts. *J.Lipid Res.* 2001;42:501-8.
93. de Antueno RJ, Knickle LC, Smith H et al. Activity of human Delta5 and Delta6 desaturases on multiple n-3 and n-6 polyunsaturated fatty acids. *FEBS Lett.* 2001;509:77-80.
94. Voss A, Reinhart M, Sankarappa S, Sprecher H. The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10, 13,16,19-docosahexaenoic acid in rat liver is independent of a 4- desaturase. *J.Biol.Chem.* 1991;266:19995-20000.
95. D'andrea S, Guillou H, Jan S et al. The same rat Delta6-desaturase not only acts on 18- but also on 24-carbon fatty acids in very-long-chain polyunsaturated fatty acid biosynthesis. *Biochem.J.* 2002;364:49-55.
96. Sprecher H. The roles of anabolic and catabolic reactions in the synthesis and recycling of polyunsaturated fatty acids. *Prostaglandins Leukot.Essent.Fatty Acids* 2002;67:79-83.
97. Martinez M. Abnormal profiles of polyunsaturated fatty acids in the brain, liver, kidney and retina of patients with peroxisomal disorders. *Brain Res.* 1992;583:171-82.
98. Martinez M, Mougan I, Roig M, Ballabriga A. Blood polyunsaturated fatty acids in patients with peroxisomal disorders. A multicenter study. *Lipids* 1994;29:273-80.
99. Ferdinandusse S, Denis S, Mooijer PAW et al. Identification of the peroxisomal {beta}-oxidation enzymes involved in the biosynthesis of docosahexaenoic acid. *J.Lipid Res.* 2001;42:1987-95.

100. Moore, S. A., Hurt, E., Yoder, Y., Sprecher, H., and Spector, A. A. Docosahexaenoic acid synthesis in human skin fibroblasts involves peroxisomal retroconversion of tetracosahexaenoic acid. *J.Lipid.Res.* 36, 2433-2443. 1995.
101. Janssen A, Baes M, Gressens P, Mannaerts GP, Declercq P, Van Veldhoven PP. Docosahexaenoic acid deficit is not a major pathogenic factor in peroxisome-deficient mice. *Lab Invest* 2000;80:31-5.
102. Sprecher H, Luthria DL, Mohammed BS, Baykousheva SP. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J.Lipid Res.* 1995;36:2471-7.
103. Sayanova O, Beaudoin F, Libisch B, Shewry P, Napier J. Mutagenesis of the borage Delta(6) fatty acid desaturase. *Biochem.Soc.Trans.* 2000;28:636-8.
104. Napier JA, Hey SJ, Lacey DJ, Shewry PR. Identification of a *Caenorhabditis elegans* Delta6-fatty-acid-desaturase by heterologous expression in *Saccharomyces cerevisiae*. *Biochem.J.* 1998;330 (Pt 2):611-4.
105. Cho HP, Nakamura MT, Clarke SD. Cloning, expression, and nutritional regulation of the mammalian Delta-6 desaturase. *J Biol Chem* 1999;274:471-7.
106. Aki T, Shimada Y, Inagaki K et al. Molecular cloning and functional characterization of rat delta-6 fatty acid desaturase. *Biochem Biophys Res Commun* 1999;255:575-9.
107. Tang C, Cho HP, Nakamura MT, Clarke SD. Regulation of human {Delta}-6 desaturase gene transcription: identification of a functional direct repeat-1 element. *J.Lipid Res.* 2003;44:686-95.
108. Gasperikova D, Demcakova E, Ukropec J, Klimes I, Sebokova E. Insulin resistance in the hereditary hypertriglyceridemic rat is associated with an impairment of delta-6 desaturase expression in liver. *Ann.N.Y.Acad.Sci.* 2002;967:446-53.
109. Sato M, Adan Y, Shibata K, Shoji Y, Sato H, Imaizumi K. Cloning of rat delta 6-desaturase and its regulation by dietary eicosapentaenoic or docosahexaenoic acid. *World Rev.Nutr.Diet.* 2001;88:196-9.
110. Jan S, Guillou H, D'andrea S et al. Myristic acid increases delta6-desaturase activity in cultured rat hepatocytes. *Reprod.Nutr.Dev.* 2004;44:131-40.
111. Gronn M, Christensen E, Hagve TA, Christophersen BO. Effects of dietary purified eicosapentaenoic acid (20:5 (n-3)) and docosahexaenoic acid (22:6(n-3)) on fatty acid desaturation and oxidation in isolated rat liver cells. *Biochim.Biophys.Acta* 1992;1125:35-43.
112. Geiger M, Mohammed BS, Sankarappa S, Sprecher H. Studies to determine if rat liver contains chain-length specific acyl-CoA 6-desaturases. *Biochim.Biophys.Acta* 1993;1170:137-42.

113. Rodriguez A, Sarda P, Nessmann C, Boulot P, Leger CL, Descomps B. Delta6- and delta5-desaturase activities in the human fetal liver: kinetic aspects. *J Lipid Res.* 1998;39:1825-32.
114. Kawashima Y, Musoh K, Kozuka H. Peroxisome proliferators enhance linoleic acid metabolism in rat liver. Increased biosynthesis of omega 6 polyunsaturated fatty acids. *J.Biol.Chem.* 1990;265:9170-5.
115. Shoji Y, Sanekata A, Sato M, Imaizumi K. Preparation of antiserum against rat delta6-desaturase and its use to evaluate the desaturase protein levels in rats treated with gemfibrozil, a ligand for peroxisome proliferator-activated receptor alpha. *Biosci.Biotechnol.Biochem.* 2003;67:1177-8.
116. Clarke SD, Jump DB. Dietary polyunsaturated fatty acid regulation of gene transcription. *Annu.Rev.Nutr.* 1994;14:83-98.
117. Kliewer SA, Sundseth SS, Jones SA et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc.Natl.Acad.Sci.U.S.A* 1997;94:4318-23.
118. Smith SA. Peroxisome proliferator-activated receptors and the regulation of mammalian lipid metabolism. *Biochem.Soc.Trans.* 2002;30:1086-90.
119. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr.Rev.* 1999;20:649-88.
120. Lawrence JW, Li Y, Chen S et al. Differential gene regulation in human versus rodent hepatocytes by peroxisome proliferator-activated receptor (PPAR) alpha. PPAR alpha fails to induce peroxisome proliferation-associated genes in human cells independently of the level of receptor expression. *J Biol.Chem.* 2001;276:31521-7.
121. Cho HP, Nakamura M, Clarke SD. Cloning, expression, and fatty acid regulation of the human delta-5 desaturase. *J Biol Chem* 1999;274:37335-9.
122. Zolfaghari R, Cifelli CJ, Banta MD, Ross AC. Fatty acid delta(5)-desaturase mRNA is regulated by dietary vitamin A and exogenous retinoic acid in liver of adult rats. *Arch.Biochem.Biophys.* 2001;391:8-15.
123. Watts JL, Browse J. Isolation and characterization of a Delta 5-fatty acid desaturase from *Caenorhabditis elegans*. *Arch.Biochem.Biophys.* 1999;362:175-82.
124. Raetz CR. Molecular genetics of membrane phospholipid synthesis. *Annu.Rev.Genet.* 1986;20:253-95.
125. Zinser E, Sperka-Gottlieb CD, Fasch EV, Kohlwein SD, Paltauf F, Daum G. Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. *J.Bacteriol.* 1991;173:2026-34.

126. Kennedy EP. The synthesis of cytidine diphosphate choline, cytidine diphosphate ethanolamine, and related compounds. *J.Biol.Chem.* 1956;222:185-91.
127. Henneberry AL, McMaster CR. Cloning and expression of a human choline/ethanolaminephosphotransferase: synthesis of phosphatidylcholine and phosphatidylethanolamine. *Biochem.J.* 1999;339 (Pt 2):291-8.
128. Adkisson HD, Risener FS, Jr., Zarrinkar PP, Walla MD, Christie WW, Wuthier RE. Unique fatty acid composition of normal cartilage: discovery of high levels of n-9 eicosatrienoic acid and low levels of n-6 polyunsaturated fatty acids. *FASEB J.* 1991;5:344-53.
129. Barcelo-Coblijn G, Collison LW, Jolly CA, Murphy EJ. Dietary alpha-linolenic acid increases brain but not heart and liver docosahexaenoic acid levels. *Lipids* 2005;40:787-98.
130. Watkins SM, Lin TY, Davis RM et al. Unique phospholipid metabolism in mouse heart in response to dietary docosahexaenoic or alpha-linolenic acids. *Lipids* 2001;36:247-54.
131. Chanmugam PS, Boudreau MD, Hwang DH. Dietary (*n*-3) fatty acids alter fatty acid composition and prostaglandin synthesis in rat testis. *J.Nutr.* 1991;121:1173-8.
132. Luthria DL, Mohammed BS, Sprecher H. Regulation of the biosynthesis of 4,7,10,13,16,19-docosahexaenoic acid. *J Biol Chem* 1996;271:16020-5.
133. Ferdinandusse S, Denis S, Dacremont G, Wanders RJ. Studies on the metabolic fate of n-3 polyunsaturated fatty acids. *J Lipid Res.* 2003;44:1992-7.
134. Mohrhauer H, Holman RT. The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver. *J.Lipid Res.* 1963;4:151-9.
135. Lands WEM, Morris A, Libelt B. Quantitative effects of dietary polyunsaturated fats on the composition of fatty acids in rat tissues. *Lipids* 1990;25:505-16.
136. Morise A, Combe N, Boue C et al. Dose effect of alpha-linolenic acid on PUFA conversion, bioavailability, and storage in the hamster. *Lipids* 2004;39:325-34.
137. Gibson RA, Neumann MA, Burnard SL, Rinaldi JA, Patten GS, McMurchie EJ. The effect of dietary supplementation with eicosapentaenoic acid on the phospholipid and fatty acid composition of erythrocytes of marmoset. *Lipids* 1992;27:169-76.
138. McMurchie EJ, Burnard SL, Rinaldi JA, Patten GS, Neumann MA, Gibson RA. Cardiac membrane lipid composition and adenylate cyclase activity following dietary eicosapentaenoic acid supplementation in the marmoset monkey. *J.Nutr.Biochem.* 1992;3:13-22.

139. Blank C, Neumann MA, Makrides M, Gibson RA. Optimizing DHA levels in piglets by lowering the linoleic acid to alpha-linolenic acid ratio. *J Lipid Res* 2002;43:1537-43.
140. Gibson RA, James MJ, Neumann MA, Hawkes JS, Cleland LG. Incorporation of dietary oleate, linoleate, α -linolenate and eicosapentaenoate into the plasma lipid fractions of four strains of rat. *Biochim.Biophys.Acta* 1992;1126:49-52.
141. Mantzioris E, James MJ, Gibson RA, Cleland LG. Differences exist in the relationships between dietary linoleic and α -linolenic acids and their respective long-chain metabolites. *Am.J.Clin.Nutr.* 1995;61:320-4.
142. Burdge GC, Calder PC. Dietary [alpha]-linolenic acid and health-related outcomes: a metabolic perspective. *Nutrition Research Reviews* 2006;19:26-52.
143. Emken EA, Adlof RO, Gulley RM. Dietary linoleic acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young adult males. *Biochim.Biophys.Acta* 1994;1213:277-88.
144. de Groot RH, Hornstra G, van Houwelingen AC, Roumen F. Effect of alpha-linolenic acid supplementation during pregnancy on maternal and neonatal polyunsaturated fatty acid status and pregnancy outcome. *Am.J Clin Nutr* 2004;79:251-60.
145. Li D, Sinclair A, Wilson A et al. Effect of dietary alpha-linolenic acid on thrombotic risk factors in vegetarian men. *Am J Clin Nutr* 1999;69:872-82.
146. Vermunt SH, Mensink RP, Simonis AM, Hornstra G. Effects of age and dietary n-3 fatty acids on the metabolism of [13C]-alpha-linolenic acid. *Lipids* 1999;34 Suppl:S127.
147. Pawlosky RJ, Hibbeln JR, Lin Y et al. Effects of beef- and fish-based diets on the kinetics of n-3 fatty acid metabolism in human subjects. *Am.J Clin Nutr* 2003;77:565-72.
148. Emken EA, Adlof RO, Duval SM, Nelson GJ. Effect of dietary docosahexaenoic acid on desaturation and uptake in vivo of isotope-labeled oleic, linoleic, and linolenic acids by male subjects. *Lipids* 1999;34:785-91.
149. Emken EA, Adlof RO, Duval SM, Nelson GJ. Effect of dietary arachidonic acid on metabolism of deuterated linoleic acid by adult male subjects. *Lipids* 1998;33:471-80.
150. Giltay EJ, Gooren LJ, Toorians AW, Katan MB, Zock PL. Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. *Am.J Clin.Nutr.* 2004;80:1167-74.
151. Vermunt SH, Mensink RP, Simonis MM, Hornstra G. Effects of dietary alpha-linolenic acid on the conversion and oxidation of 13C-alpha-linolenic acid. *Lipids* 2000;35:137-42.

152. Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to palmitic, palmitoleic, stearic and oleic acids in men and women. *Prostaglandins Leukot.Essent.Fatty Acids* 2003;69:283-90.
153. James MJ, Ursin VM, Cleland LG. Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *Am J Clin Nutr* 2003;77:1140.
154. Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. *Br.J Nutr* 2003;89:679-89.
155. Harper CR, Edwards MJ, DeFilipis AP, Jacobson TA. Flaxseed oil increases the plasma concentrations of cardioprotective (n-3) fatty acids in humans. *J.Nutr.* 2006;136:83-7.
156. Goyens PL, Spilker ME, Zock PL, Katan MB, Mensink RP. Conversion of alpha-linolenic acid in humans is influenced by the absolute amounts of alpha-linolenic acid and linoleic acid in the diet and not by their ratio. *Am.J.Clin.Nutr.* 2006;84:44-53.
157. Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N, Jr. Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. *J Lipid Res.* 2001;42:1257-65.
158. de Antueno RJ, Knickle LC, Smith H et al. Activity of human Delta5 and Delta6 desaturases on multiple n-3 and n-6 polyunsaturated fatty acids. *FEBS Lett.* 2001;509:77-80.
159. Chen Q, Nilsson A. Desaturation and chain elongation of n - 3 and n - 6 polyunsaturated fatty acids in the human CaCo-2 cell line. *Biochim.Biophys.Acta* 1993;1166:193-201.
160. Dokko RC, Cho BH, Chung BH. Cellular uptake of stearic, oleic, linoleic, and linolenic acid and their effects on synthesis and secretion of lipids in Hep-G2 cells. *Int.J.Biochem.Cell Biol.* 1998;30:65-76.
161. Marra CA, De Alaniz MJ. Incorporation and metabolic conversion of saturated and unsaturated fatty acids in SK-Hep1 human hepatoma cells in culture. *Mol.Cell Biochem.* 1992;117:107-18.
162. Melin T, Nilsson A. Delta-6-desaturase and delta-5-desaturase in human Hep G2 cells are both fatty acid interconversion rate limiting and are upregulated under essential fatty acid deficient conditions. *Prostaglandins Leukot.Essent.Fatty Acids* 1997;56:437-42.
163. Angeletti C, De Alaniz MJ. Fatty acid uptake and metabolism in Hep G2 human-hepatoma cells. *Mol.Cell Biochem.* 1995;143:99-105.
164. Nara TY, He WS, Tang C, Clarke SD, Nakamura MT. The E-box like sterol regulatory element mediates the suppression of human Delta-6 desaturase gene by highly unsaturated fatty acids. *Biochem.Biophys.Res.Commun.* 2002;296:111-7.

165. Gherardi E, Thomas K, Le Cras TD, Fitzsimmons C, Moorby CD, Bowyer DE. Growth requirements and expression of LDL receptor and HMG-CoA reductase in Hep G2 hepatoblastoma cells cultured in a chemically defined medium. *J.Cell Sci.* 1992;103 (Pt 2):531-9.
166. Dixon JL, Ginsberg HN. Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. *J.Lipid Res.* 1993;34:167-79.
167. Javitt NB. Hep G2 cells as a resource for metabolic studies: lipoprotein, cholesterol, and bile acids. *FASEB J.* 1990;4:161-8.
168. Clayton RF, Rinaldi A, Kandyba EE et al. Liver cell lines for the study of hepatocyte functions and immunological response. *Liver Int.* 2005;25:389-402.
169. van Pelt JF, Decorte R, Yap PS, Fevery J. Identification of HepG2 variant cell lines by short tandem repeat (STR) analysis. *Mol.Cell Biochem.* 2003;243:49-54.
170. Chilton FH, Connell TR. 1-ether-linked phosphoglycerides. Major endogenous sources of arachidonate in the human neutrophil. *J.Biol.Chem.* 1988;263:5260-5.
171. Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J.Biol.Chem.* 1957;226:497-509.
172. Nichols PD, Danaher KT, Koslow JA. Occurrence of high levels of tetracosahexaenoic acid in the jellyfish *Aurelia* sp. *Lipids* 2003;38:1207-10.
173. Fellenberg AJJDWPAaSP. Simple mass spectrometric differentiation of the *n*-3, *n*-6 and *n*-9 series of methylene interrupted polyenoic acids. *Biological Mass Spectrometry* 1987;14:127-9.
174. Sanders TAB. Dietary fat and platelet function. *Clin.Sci.* 1983;65:343-50.
175. Kris-Etherton PM, Taylor DS, Yu-Poth S et al. Polyunsaturated fatty acids in the food chain in the United States. *Am.J Clin.Nutr.* 2000;71:179S-88S.
176. Innis SM, Elias SL. Intakes of essential *n*-6 and *n*-3 polyunsaturated fatty acids among pregnant Canadian women. *Am.J.Clin.Nutr.* 2003;77:473-8.
177. Sprecher H, Chen Q, Yin FQ. Regulation of the biosynthesis of 22:5*n*-6 and 22:6*n*-3: a complex intracellular process. *Lipids* 1999;34 Suppl:S153-S156.
178. Sprecher H, Chen Q. Polyunsaturated fatty acid biosynthesis: a microsomal-peroxisomal process. *Prostaglandins Leukot Essent Fatty Acids* 1999;60:317-21.
179. Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of *n*-3 fatty acids in humans. *Am.J.Clin.Nutr.* 2006;83:1467S-76S.

180. McMurchie EJ, Rinaldi JA, Burnard SL et al. Incorporation and effects of dietary eicosapentaenoate (20:5(*n*-3)) on plasma and erythrocyte lipids of the marmoset following dietary supplementation with differing levels of linoleic acid. *Biochim.Biophys.Acta* 1990;1045:164-73.
181. Langelier B, Alessandri JM, Perruchot MH, Guesnet P, Lavalie M. Changes of the transcriptional and fatty acid profiles in response to *n*-3 fatty acids in SH-SY5Y neuroblastoma cells. *Lipids* 2005;40:719-28.
182. Moore SA, Yoder E, Spector AA. Role of the blood-brain barrier in the formation of long chain *n*-3 and *n*-6 fatty acids from essential fatty acid precursors. *J.Neurochem.* 1990;55:391-402.
183. Delton-Vandenbroucke I, Grammas P, Anderson RE. Polyunsaturated fatty acid metabolism in retinal and cerebral microvascular endothelial cells. *J Lipid Res.* 1997;38:147-59.
184. Makrides M, Neumann MA, Byard RW, Simmer K, Gibson RA. Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. *Am.J.Clin.Nutr.* 1994;60:189-94.
185. Lefkowitz W, Lim SY, Lin Y, Salem N, Jr. Where does the developing brain obtain its docosahexaenoic acid? Relative contributions of dietary alpha-linolenic acid, docosahexaenoic acid, and body stores in the developing rat. *Pediatr.Res.* 2005;57:157-65.
186. Mori TA, Burke V, Puddey IB et al. Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am.J.Clin.Nutr.* 2000;71:1085-94.
187. Woodman RJ, Mori TA, Burke V, Puddey IB, Watts GF, Beilin LJ. Effects of purified eicosapentaenoic and docosahexaenoic acids on glycemic control, blood pressure, and serum lipids in type 2 diabetic patients with treated hypertension. *Am J Clin Nutr* 2002;76:1007-15.
188. Nestel P, Shige H, Pomeroy S, Cehun M, Abbey M, Raederstorff D. The *n*-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid increase systemic arterial compliance in humans. *Am J Clin Nutr* 2002;76:326-30.
189. Grimsgaard S, Bonna KH, Hansen JB, Nordoy A. Highly purified eicosapentaenoic acid and docosahexaenoic acid in humans have similar triacylglycerol-lowering effects but divergent effects on serum fatty acids. *Am.J.Clin.Nutr.* 1997;66:649-59.
190. Baykousheva SP, Luthria DL, Sprecher H. Arachidonic acid formed by peroxisomal beta-oxidation of 7,10,13,16-docosatetraenoic acid is esterified into 1-acyl-sn-glycero-3-phosphocholine by microsomes. *J Biol.Chem.* 1994;269:18390-4.
191. Baykousheva SP, Luthria DL, Sprecher H. Peroxisomal-microsomal communication in unsaturated fatty acid metabolism. *FEBS Lett.* 1995;367:198-200.

192. Jamil H, Utal AK, Vance DE. Evidence that cyclic AMP-induced inhibition of phosphatidylcholine biosynthesis is caused by a decrease in cellular diacylglycerol levels in cultured rat hepatocytes. *J.Biol.Chem.* 1992;267:1752-60.
193. Choy PC, Skrzypczak M, Lee D, Jay FT. Acyl-GPC and alkenyl/alkyl-GPC:acyl-CoA acyltransferases. *Biochim.Biophys.Acta* 1997;1348:124-33.
194. Choy PC, Arthur G. In: Vance DE, ed. *Phosphatidylcholine metabolism*. Boca Raton: CRC Press 1989:87-101.
195. Mohammed BS, Luthria DL, Bakousheva SP, Sprecher H. Regulation of the biosynthesis of 4,7,10,13,16-docosapentaenoic acid. *Biochem J* 1997;326 (Pt 2):425-30.
196. Chen J, Rider DA, Ruan R. Identification of valid housekeeping genes and antioxidant enzyme gene expression change in the aging rat liver. *J.Gerontol.A Biol.Sci.Med.Sci.* 2006;61:20-7.
197. de Kok JB, Roelofs RW, Giesendorf BA et al. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Lab Invest* 2005;85:154-9.
198. Marquardt A, Stohr H, White K, Weber BH. cDNA cloning, genomic structure, and chromosomal localization of three members of the human fatty acid desaturase family. *Genomics* 2000;66:175-83.
199. Smith PK, Krohn RI, Hermanson GT et al. Measurement of protein using bicinchoninic acid. *Anal.Biochem.* 1985;150:76-85.
200. Wiechelman KJ, Braun RD, Fitzpatrick JD. Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Anal.Biochem.* 1988;175:231-7.
201. Lekanne Deprez RH, Fijnvandraat AC, Ruijter JM, Moorman AF. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Anal.Biochem.* 2002;307:63-9.
202. Steineger HH, Sorensen HN, Tugwood JD, Skrede S, Spydevold O, Gautvik KM. Dexamethasone and insulin demonstrate marked and opposite regulation of the steady-state mRNA level of the peroxisomal proliferator-activated receptor (PPAR) in hepatic cells. Hormonal modulation of fatty-acid-induced transcription. *Eur.J.Biochem.* 1994;225:967-74.
203. Saether T, Tran TN, Rootwelt H, Christophersen BO, Haugen TB. Expression and regulation of delta5-desaturase, delta6-desaturase, stearyl-coenzyme A (CoA) desaturase 1, and stearyl-CoA desaturase 2 in rat testis. *Biol.Reprod.* 2003;69:117-24.
204. Matsuzaka T, Shimano H, Yahagi N et al. Dual regulation of mouse Delta(5)- and Delta(6)-desaturase gene expression by SREBP-1 and PPARalpha. *J.Lipid Res.* 2002;43:107-14.

205. Song HW, Nara TY, Nakamura MT. Delayed induction of delta-6 and delta-5 desaturases by a peroxisome proliferator. *Biochem.Biophys.Res.Commun.* 2002;299:832-8.
206. Hsu MH, Savas U, Griffin KJ, Johnson EF. Identification of peroxisome proliferator-responsive human genes by elevated expression of the peroxisome proliferator-activated receptor alpha in HepG2 cells. *J Biol.Chem.* 2001;276:27950-8.
207. Burdge GC. Metabolism of alpha-linolenic acid in humans. *Prostaglandins Leukot.Essent.Fatty Acids* 2006.
208. Obukowicz MG, Welsch DJ, Salsgiver WJ et al. Novel, selective delta6 or delta5 fatty acid desaturase inhibitors as antiinflammatory agents in mice. *J Pharmacol.Exp.Ther.* 1998;287:157-66.
209. Harmon SD, Kaduce TL, Manuel TD, Spector AA. Effect of the delta6-desaturase inhibitor SC-26196 on PUFA metabolism in human cells. *Lipids* 2003;38:469-76.
210. Jones BH, Maher MA, Banz WJ et al. Adipose tissue stearoyl-CoA desaturase mRNA is increased by obesity and decreased by polyunsaturated fatty acids. *Am.J.Physiol* 1996;271:E44-E49.
211. Sauerwald TU, Hachey DL, Jensen CL, Chen HM, Anderson RE, Heird WC. Intermediates in endogenous synthesis of C22:6 omega 3 and C20: 4 omega 6 by term and preterm infants. *Pediatr.Res.* 1997;41:183-7.
212. Wanders RJ, Vreken P, Ferdinandusse S et al. Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochem.Soc.Trans.* 2001;29:250-67.
213. Rosenthal MD. Fatty acid metabolism of isolated mammalian cells. *Prog.Lipid Res.* 1987;26:87-124.
214. Gordon JA, Heller SK, Kaduce TL, Spector AA. Formation and release of a peroxisome-dependent arachidonic acid metabolite by human skin fibroblasts. *J Biol.Chem.* 1994;269:4103-9.
215. Williard DE, Kaduce TL, Harmon SD, Spector AA. Conversion of eicosapentaenoic acid to chain-shortened omega-3 fatty acid metabolites by peroxisomal oxidation. *J Lipid Res.* 1998;39:978-86.
216. Retterstol K, Haugen TB, Christophersen BO. The pathway from arachidonic to docosapentaenoic acid (20:4n-6 to 22:5n-6) and from eicosapentaenoic to docosahexaenoic acid (20:5n-3 to 22:6n-3) studied in testicular cells from immature rats. *Biochim.Biophys.Acta* 2000;1483:119-31.
217. Reddy JK, Hashimoto T. Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annu.Rev.Nutr* 2001;21:193-230.

218. Khan SA, Vanden Heuvel JP. Role of nuclear receptors in the regulation of gene expression by dietary fatty acids (review). *J Nutr Biochem*. 2003;14:554-67.
219. Tian Q, Grzemeski FA, Panagiotopoulos S, Ahokas JT. Peroxisome proliferator-activated receptor alpha agonist, clofibrate, has profound influence on myocardial fatty acid composition. *Chem.Biol.Interact*. 2006;160:241-51.
220. Li Y, Nara TY, Nakamura MT. Peroxisome proliferator-activated receptor alpha is required for feedback regulation of highly unsaturated fatty acid synthesis. *J Lipid Res*. 2005;46:2432-40.
221. Delany JP, Windhauser MM, Champagne CM, Bray GA. Differential oxidation of individual dietary fatty acids in humans. *Am.J Clin Nutr* 2000;72:905-11.
222. Brenna JT. Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man. *Curr.Opin.Clin Nutr Metab.Care* 2002;5:127-32.
223. Burdge GC, Calder PC. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod.Nutr Dev*. 2005;45:581-97.
224. Irving CS, Wong WW, Shulman RJ, Smith EO, Klein PD. [13C]bicarbonate kinetics in humans: intra- vs. interindividual variations. *Am.J.Physiol* 1983;245:R190-R202.
225. Clouet P, Niot I, Bezard J. Pathway of alpha-linolenic acid through the mitochondrial outer membrane in the rat liver and influence on the rate of oxidation. Comparison with linoleic and oleic acids. *Biochem.J* 1989;263:867-73.
226. Howe PR, Downing JA, Grenyer BF, Grigonis-Deane EM, Bryden WL. Tuna fishmeal as a source of DHA for n-3 PUFA enrichment of pork, chicken, and eggs. *Lipids* 2002;37:1067-76.
227. Vidgren HM, Agren JJ, Schwab U, Rissanen T, Hanninen O, Uusitupa MI. Incorporation of n-3 fatty acids into plasma lipid fractions, and erythrocyte membranes and platelets during dietary supplementation with fish, fish oil, and docosahexaenoic acid-rich oil among healthy young men. *Lipids* 1997;32:697-705.
228. Tugwood JD, Aldridge TC, Lambe KG, Macdonald N, Woodyatt NJ. Peroxisome proliferator-activated receptors: structures and function. *Ann.N Y.Acad.Sci*. 1996;804:252-65.
229. Tugwood JD, Aldridge TC, Lambe KG, Macdonald N, Woodyatt NJ. Peroxisome proliferator-activated receptor-alpha and the pleiotropic responses to peroxisome proliferators. *Arch.Toxicol.Suppl* 1998;20:377-86.

230. Palmer CN, Hsu MH, Griffin KJ, Raucy JL, Johnson EF. Peroxisome proliferator activated receptor-alpha expression in human liver. *Mol.Pharmacol.* 1998;53:14-22.
231. Wanders RJ, van Roermund CW, Griffioen M, Cohen L. Peroxisomal enzyme activities in the human hepatoblastoma cell line HepG2 as compared to human liver. *Biochim.Biophys.Acta* 1991;1115:54-9.
232. Gottlicher M, Widmark E, Li Q, Gustafsson JA. Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc.Natl.Acad.Sci.U.S.A* 1992;89:4653-7.