THESIS TITLE:

Effects of dietary fish oil and fibre on contractility

of gut smooth muscle

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ABSTRACT

From animal experimentation, and studies using in vitro models, there was evidence in the literature to suggest that dietary fibre may influence contractility and motility of the gastrointestinal tract and long chain (LC) n-3 polyunsaturated fatty acids (PUFAs) from marine sources may influence contractility of smooth muscle cells in blood vessels. The hypothesis of this thesis was that dietary fish oil and/or fibre influence the contractility of isolated intact sections of gut smooth muscle tissue from small animal models. Methodology was established to measure in vitro contractility of intact pieces of guinea pig ileum with the serosal side isolated from the lumen. It was demonstrated that four amino acid peptides from κ -casein (casoxins) applied to the lumen overcame morphine-induced inhibition of contraction. Using this established technology, the guinea pig was used to investigate the effects of dietary fibre and fish oil supplementation on gut *in vitro* contractility. In separate experiments, changes in sensitivity to electrically-driven and 8-*iso*-prostanglandin (PG)E₂-induced contractility were demonstrated for dietary fibre and fish oil. A modified, isolated gut super-perfusion system was then established for the rat to validate these findings. It was subsequently shown that LC n-3 PUFA from dietary fish oil significantly increased maximal contraction in response to the G-protein coupled receptor modulators, acetylcholine and the eicosanoids PGE_2 , $PGF_{2\alpha}$, 8-iso-PGE₂ and U-46619 in ileum but not colon, without changes in sensitivity (EC_{50}), when n-3 PUFA as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) had been incorporated to a similar degree into the gut total phospholipid membrane pool. It was further established that the spontaneously hypertensive rat (SHR) had a depressed prostanoid $(PGE_2 \text{ and } PGF_{2\alpha})$ response in the gut that could be restored by dietary fish oil supplementation (5% w/w of total diet) in the ileum but not the colon. Importantly,

the muscarinic response in the colon of the SHR was increased by fish oil supplementation with DHA likely to be the active agent. Dietary fish oil dose experiments deduced differential increases in response occurred at fish oil concentrations of 1% for muscarinic and 2.5% (w/w) for prostanoid stimulators of the ileum with no difference in receptor-independent KCl-induced depolarization-driven contractility. Studies combining high amylose resistant starch (HAMS, 10% w/w) and fish oil (10% w/w) fed to young rats demonstrated a low prostanoid response that was enhanced by dietary fish oil but not resistant starch. There was however, an interactive effect of the HAMS and fish oil noted for the muscarinic-mimetic, carbachol. Generally, resistant starch increased the large bowel short chain fatty acid pool with a subsequent lower pH. Binding studies determined that while the total muscarinic receptor binding properties of an isolated ileal membrane fraction were not affected in mature rats by dietary fish oil, young rats had a different order of muscarinic receptor subtype response with a rank order potency of $M_3 > M_1 > M_2$ compared to mature animals of $M_3 > M_2 > M_1$ with fish oil altering the sensitivity of the M_1 receptor subtype in isolated carbachol-precontracted ileal tissue. In conclusion, experiments using the guinea pig and rat gut models demonstrated that dietary fish oil supplementation, and to a lesser degree fibre, increased receptor-driven contractility in normal and compromised SHR ileum and colon. Further, changes in responsiveness were demonstrated in the developing rat gut prostanoid and muscarinic receptor populations that could be altered by dietary fish oil. Preliminary evidence suggested that fish oil as DHA may alter receptor-driven gut contractility by mechanisms involving smooth muscle calcium modulation. Defining the role that dietary fibre and fish oil, and other nutrients, play in normal and diseased states of bowel health such as

inflammatory bowel disease (IBD), where contractility is compromised, are among the ongoing challenges.



DECLARATION:

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.....

Glen Stephen Patten (August 2007)

"All great truths begin as blasphemies." George Bernard Shaw

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I dedicate this thesis to my late parents, father, Frank and mother, Dulcie, and siblings, Arthur, Bruce (deceased) and Louise. Finally, to Sue Hicks, all my dearest thanks for your support, love, and encouragement past, now and in the future.

LIST OF PUBLICATIONS AND ABSTRACTS THAT CONTRIBUTED TO THIS THESIS

Full papers

- 1. **Patten GS**, Head RJ, Abeywardena MY and McMurchie EJ. (2001) An apparatus to assay opioid activity in the infused lumen of the intact isolated guinea pig ileum. *J Pharmacol Toxicol Methods*, **45**: 39-46.
- 2. **Patten GS**, Bird AR, Topping DL and Abeywardena MY. (2002a) Dietary fish oil alters the sensitivity of guinea pig ileum to electrical driven contractions and 8-*iso*-PGE₂. *Nutr Res*, **22**: 1413-26.
- 3. **Patten GS**, Abeywardena MY, McMurchie EJ and Jahangiri A. (2002b) Dietary fish oil increases acetylcholine- and eicosanoid-induced contractility of isolated rat ileum. *J Nutr*, **132**: 2506-13.
- 4. **Patten GS**, Bird AR, Topping DL and Abeywardena MY (2004a) Effects of convenience rice congee supplemented diets on guinea pig whole animal and gut growth, caecal digesta SCFA and in vitro ileal contractility. *Asia Pac J Clin Nutr*, **13**: 92-100.
- 5. **Patten GS**, Adams MJ, Dallimore JA and Abeywardena MY (2004b) Depressed prostanoid-induced contractility of the gut in spontaneously hypertensive rats (SHR) is not affected by the level of dietary fat. *J Nutr*, **134**: 2924-29.
- 6. **Patten GS**, Adams MJ, Dallimore JA, Rogers PF, Topping DL and Abeywardena MY (2005a) Restoration of depressed prostanoid-induced ileal contraction in spontaneously hypertensive rats by dietary fish oil. *Lipids*, **40**: 69-79.
- 7. **Patten GS**, Adams MJ, Dallimore JA and Abeywardena MY (2005b) Dietary fish oil dose-response effects on ileal phospholipid fatty acids and contractility. *Lipids*, **40**:925-29.
- 8. **Patten GS**, Conlon MA, Bird AR, Adams MJ, Topping DL and Abeywardena MY (2006) Interactive effects of dietary resistant starch and fish oil on SCFA production and agonist-induced contractility in ileum of young rats. *Dig Dis Sci*, **51**:254-61.

Abstracts

1. **Patten GS**, McMurchie EJ, Abeywardena MY and Jahangiri A (2001) Dietary fish oil supplementation increases the in vitro contractility of rat ileum. NSA, Canberra, Dec 3-5. *Asia Pac J Clin Nutr*, **10** Suppl: S10.

- Patten GS and Abeywardena MY (2003) Dietary n-3 PUFA on indices of bowel health. GESA, Adelaide Meeting. J Gastroenterol Hepatology, 17 [Suppl.]: 160.
- 3. **Patten GS** and Abeywardena MY (2003) Fish oil feeding increases gut contractility in spontaneous hypertensive rat (SHR) model. NSA, Hobart, Nov 30 Dec 3. *Asia Pac J Clin Nutr*, **12** [Suppl]: S64.
- 4. **Patten GS** and Abeywardena MY (2003) High saturated fat diet does not affect gut contractility in the rat. NSA, Hobart, Nov 30 Dec 3. *Asia Pac J Clin Nutr*, **12** [Suppl]: S65.
- 5. **Patten GS** and Abeywardena MY (2004) Role for long chain n-3 PUFA in gut contractility. AOCS Australasian Section. Biennial Workshop. Fats and oils their role in food and health. Adelaide Nov 30 Dec 1.



STATEMENT OF JOINTLY AUTHORED PAPERS ON THE CONTRIBU-TIONS MADE BY EACH AUTHOR AS LISTED.

Glen S. Patten was involved in the study design and formulation of dietary regimens for papers (as listed above) 1, 2, 4, 5, 6, and 7, and designed and undertook all of the *in vitro* gut contractility studies and was the principal and corresponding author of all papers (1-8).

Richard J. Head assisted in the experimental design to paper 1 and assisted in the manuscript draft.

Mahinda Y. Abeywardena was involved in study design for papers 1, 5, 6, and 7 and assisted in the drafting of papers.

Edward J. McMurchie was involved with study design for papers 1 and 3 and assisted in the manuscript draft.

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Anisa Jahangiri was involved with study design of paper 3 and assisted in the manuscript draft.

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Julie A. Dallimore was involved with study design for papers 5, 6, and 7, and assisted in manuscript draft.

Paul F. Rogers assisted with muscarinic receptor binding studies for paper 6 and assisted with drafting of the manuscript.

Michael A. Conlon was involved with study design for paper 8 and assisted in the manuscript draft.

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ABBREVIATIONS

AA – Arachidonic acid

ALA – Alpha linolenic acid

Ang II – Angiotensin II

ATP- Adenosine triphosphate

B_{max} – Maximal amount of binding that would occur

 Ca^{2+} - Calcium ion

 $[Ca^{2+}]_i$ – Intracellular calcium ion concentration

CCh - Carbachol

CCK- Cholecystokinin

CD - Crohn's disease

Cdk2 - Cyclin-dependent kinase-2

CHN – CSIRO Human Nutrition

CLA - Conjugated linoleic acid

COX - Cyclooxygenase

CSIRO - Commonwealth Scientific and Industrial Research Organisation

CVD – Cardiovascular disease

 $\Delta \Psi m$ - Mitochondrial transmembrane potential

DHA – Docosahexaenoic acid

DPA – Docosapentaenoic acid

EC - Enterochromaffin cell

EC₅₀ – Effective concentration at which half the maximal biological effect is achieved

EDHF - Endothelium derived hyperpolarising factor

EPA – Eicosapentaenoic acid

FA(s) - Fatty acid(s)

FO - Fish oil

GIT – Gastrointestinal tract

GLA – Gamma linolenic acid

HAMS -High amylose maize starch

5-HEPE - 5-Hydroxyeicosapentaenoic acid

5-HETE – 5-Hydroxyeicosatetraenoic acid

5-HPETE – 5-Hydroperoxyeicosatetraenoic acid

5-HT – 5-Hydroxytryptamine, serotonin

 IC_{50} – Inhibitory concentration at which 50% of the biological effect occurs

ICAM-1 – Intercellular adhesion molecule-1

IBD - Inflammatory bowel disease

I_{cat} - Non-selective cation current

 $I\kappa B$ - Inhibitory binding protein- κB

IL-1 - Interleukin-1

8-*iso*-PGE₂ – 8-*iso*prostaglandin E₂

 K_d – The concentration at which 50% of maximal binding has occurred

LA – Linoleic acid

LC – Long chain

LDL – Low density lipoprotein

LOX - Lipoxygenase

LT - Leukotriene

NF- κ B - Nuclear factor κ B

PDGF - Platelet derived growth factor

NO - Nitric oxide

iNOS – Nitric oxide synthetase (inducible) OO - Olive oil PD1 - Protectin D1 PDGF – Platelet derived growth factor 5-PEPE - 5-Per(oxy)eicosapentaenoic acid PG - Prostaglandin $PGE_2 - Prostaglandin E_2$ $PGF_{2\alpha}$ - Prostaglandin $F_{2\alpha}$ $PGH_2 - Prostaglandin H_2$ PGI₂ – Prostaglandin I₂ PHGG – Partially hydrolysed guar gum PKC - Protein kinase C PL - Phospholipid PMN - Polymorphonuclear leukocytes PPARy - Peroxisome proliferator-activated receptor gamma PS - Phosphatidylserine PUFA(s) – Polyunsaturated fatty acid(s) PYY - Peptide YY ROS - Reactive oxygen species rhIL-11 – Human recombinant IL-11 RS – Resistant starch SCFA(s) – Short chain fatty acid(s) SD – Sprague -Dawley SD – Standard deviation SEM – Standard error of the mean SF - Saturated fat SFA – Saturated fatty acid SHR – Spontaneously hypertensive rat SMC – Smooth muscle cell SO - Sunflower (or safflower oil) SREBP-1c - Sterol receptor element binding protein-1c SR - Sarcoplasmic reticulum TAG - Triacylglyceride Th – T helper cell TNF_{α} - Tumour necrosis factor-alpha TTX - Tetrodotoxin TXA_2 – Thromboxane A_2 UC - Ulcerative colitis VCAM-1 - Vascular cell adhesion molecule-1 VSMC - Vascular smooth muscle cell WKY - Wistar-Kyoto

Effects of dietary fish oil and fibre on contractility of gut smooth muscle

Literature Review

1.1. Introduction

There is an increasing body of evidence that dietary fibre is good for laxation and bowel health and that long chain (LC) n-3 polyunsaturated fatty acids (PUFAs) found in high amounts in oily fish are good for the cardiovascular system and beneficial for inflammatory conditions including those affecting gut function. Dietary fibre refers to the indigestible carbohydrates found in fruit, vegetables, grain and nuts. Fermentation of fibre in the large bowel microflora produces short chain fatty acids (SCFAs) that play a key role in bowel health (Topping and Clifton, 2001). The LC n-3 PUFAs are produced by marine microalgae and phytoplankton and eaten by krill and other small animals and so on up the food chain to man. Both SCFAs and LC n-3 PUFAs may have beneficial effects on human well being and as such represent an active area of research (Freeman, *et al.*, 2006; McDonald, 2006; Wong *et al.*, 2006; Leaf 2007).

One of the first researchers to postulate a beneficial link between a diet rich in fish fat and cardiovascular health was Hugh Sinclair in the mid twentieth century, although his hypotheses were not widely accepted at the time (Sinclair, 1956). Proof-of-concept awaited the epidemiologically-based observations on Greenland Eskimos in the 1970s by Dyerberg and Bang (Bang *et al.*, 1971) and the concomitant flow of information relating to the beneficial effects of LC PUFAs on atherosclerosis and cardiovascular disease (Schmidt *et al.*, 2006). In addition to the abovementioned conditions was also the recognition of the therapeutic effects of fish oil on inflammatory conditions and subsequent possible protection from the development of cancers, particularly of the



Figure 1. Proposed beneficial effects of dietary fish oil. The long chain (LC) polyunsaturated fatty acids (PUFA) found in high concentrations in certain oily fish have been postulated to be efficacious to various degrees in the prevention and treatment of a wide range of pathologies that generally involve underlying tissue inflammation.

bowel (De Caterina *et al.*, 1994; Roynette *et al.*, 2004; Calder, 2006; Chapkin *et al.*, 2007a,b; Mund *et al.*, 2007). The role of dietary fibre as a component of whole grain has also been postulated to correlate with lower incidence and protection from colon cancer and to possibly assist with inflammatory diseases of the gastrointestinal tract (Galvez *et al.*, 2005; Jacobs *et al.*, 2007). As yet, the case for LC n-3 PUFA in the protection and treatment for IBD is still inconclusive due to the paucity of detailed animal studies backed by large, well controlled human clinical trials (Nakazawa and Hibi, 2000; Ruxton *et al.*, 2007; Turner *et al.*, 2007a,b).

More recently, associative evidence is emerging for the health benefits of fish oil on the brain and nervous system (Conklin *et al.*, 2007) including vision (Cheatham *et al.*,

2006; Eilander *et al.*, 2007) mood and neuropsychiatric disorders (Young *et al.*, 2005; Hallahan *et al.*, 2007). The role of fish oil and other micronutrients for treatment of both inflammatory bowel disease (IBD) (MacLean *et al.*, 2005; Camuesco *et al.*, 2006; Turner *et al.*, 2007a,b), where a proportion of patients may have essential fatty acid deficiency (Siguel and Lerman, 1996; Figler *et al.*, 2007), and for the treatment of vascular complications that occur in diabetes, is also gaining some momentum (Nettleton and Katz, 2005; Freeman *et al.*, 2006; Lombardo and Chicco, 2006). Finally, there is conflicting evidence for n-3 PUFA on atopic and pulmonary inflammatory conditions such as asthma (Reisman *et al.*, 2006; Almqvist *et al.*, 2007; Hwang *et al.*, 2007). In general, populations that show lower cardiovascular disease (CVD), inflammatory diseases and neuroses have a relatively high n-3/n-6 ratio that is expressed in plasma and tissue (Simopoulos, 2002a,b). See **Figure 1** for a summary of LC n-3 PUFA effects on various inflammation-linked conditions.

1.2 Background to experimentation relating to this thesis

The focus of the experimental work of this thesis is to explore the effects of dietary fibre and fish oil on small animal gut contractility. The biological mechanisms involved with n-3 FA metabolism and effects on physiology and pathophysiology are complicated but are generally related to immunoregulatory white blood cells (leucocytes and macrophages) (Lin *et al.*, 2007) and the dietary n-3/n-6 ratio which modulates amongst other things; cell membrane properties (McMurchie and Raison, 1979; Patten *et al.*, 1989; Leifert *et al.*, 2000a,b; McLennan and Abeywardena, 2005), calcium handling and homeostasis (Nair *et al.*, 1997; Leaf, 2001), eicosanoid synthesis (Abeywardena *et al.*, 1987, 1991a,b; James *et al.*, 2000, proinflammatory cytokine production (interleukin-1 and leukotriene B₄) (Calder, 2003, 2006), and

regulation of nuclear receptors including the liver X receptor, hepatocyte nuclear factor- 4α , farnesol X receptor, and the peroxisome proliferator-activated receptors (PPARs) and effects on gene expression via, for example, sterol receptor element binding protein-1c (SREBP-1c) (Sampath and Ntambi, 2004; Nakatani *et al.*, 2005; Davidson, 2006; Gao *et al*, 2007; Nieto, 2007).

To date, there is a small amount of information about the effects of dietary fibre and some emerging evidence about the effects of fatty acids on gut physiology as it relates to contractility and motility (Jonkers *et al.*, 2003; Patten *et al.*, 2004b). To help understand the physiology involved in muscle contractility, part one of this literature review will focus on the effects of fish oil, and/or fibre, on contractility in cardiac, and smooth muscle cells of the blood vessels that are well documented and pertains in part to mechanisms which may be playing a role in gut physiology and pathophysiology. In part two, there will be an overview of the experiments published, in respect to this thesis, on the use of small animal models to investigate the effects of dietary fish oil and fibre on gut contractility. The final section of Part 2 will outline some preliminary results and speculate on potential future studies. It is not in the scope of this literature review to discuss in depth the role of fibre and LC n-3 PUFA in CVD, cancer, or IBD.

1.3. Hypothesis

From the literature, there is some evidence that dietary fish oil can influence the contractility of cardiac muscle and smooth muscle of blood vessels and dietary fibre can influence the contractility of gut tissue to varying degrees. The hypothesis of this thesis is that dietary fish oil and/or fibre influence the contractility of isolated intact sections of gut smooth muscle tissue from small animal models.

1.4. Experimental aims

- Develop *in vitro* contractility models for isolated intact gut sections of ileum and colon from the guinea pig and the rat.
- ii) Investigate the effects of dietary fibre and fish oil supplementation on gut lipid profiles and contractility outcomes in the guinea pig and rat.
- iii) Determine the contractility profile of isolated gut tissue from the spontaneously hypertensive rat (SHR) fed diets supplemented with saturated fat, canola oil or fish oil.
- iv) Characterize the dose effects of dietary fish oil on contractility of isolated gut tissue from WKY rats.
- v) Determine any interactive effects of dietary fish oil and fibre on contractility of isolated gut from normotensive rats.
- vi) Examine aspects of the muscarinic receptor signalling system to investigate possible biochemical mechanisms of dietary fish oil LC n-3
 PUFA involved in the modification of gut contractility.

1.5. Definition of fatty acids

Fatty acids (FAs) typically have an even number of carbon atoms, in the range of 2-26. FAs with only single bonds between adjacent carbon atoms are referred to as 'saturated', whereas those with at least one C=C double bond are called 'unsaturated' (Laposata, 1995) (see **Table 1** for a list of common fatty acids). The PUFAs have two or more double bonds which are usually methylene- interrupted (non-conjugated) and are named according to the position of these bonds and the total chain length. For example DHA (22:6) is an omega-3 (n-3) FA with 22 carbon atoms and six double



bonds, usually in an all *cis* configuration (see **Figure 2** and **Figure 3** for diagrammatic representations of EPA and DHA). The term 'n-3' indicates that, counting from the methyl end of the molecule, the first double bond is located between the third and fourth carbons. As the degree of unsaturation in FAs increases, the melting point decreases which confer the attribute of fluidity of n-3 PUFAs in cell membranes that influence many aspects of cell function (McMurchie and Raison, 1979; Valentine and Valentine, 2004; Ruxton *et al.*, 2007).



Figure 3. 3-D model of docosahexaenoic acid (DHA).

In nature, DHA and eicosapentaenoic acid (EPA, 20:5n-3) are produced by unicellular phytoplankton and microalgae that are ingested by smaller marine creatures such as krill and are thus found in high concentrations (up to 3% of total fish weight) in cold water species of oily fish such as herrings, sardine, salmon and mackerel (Romero *et al.*, 1996, Oh *et al.*, 2006). EPA and DHA are synthesized from the n-3 precursor α linolenic acid (ALA, 18:3n-3) whereas the long chain n-6 PUFA such as arachidonic acid (AA, 20:4n-6) are synthesized from the predominantly plant-derived precursor linoleic acid (18:2, n-6) (Jump, 2002). Plants also produce α -linolenic acid (ALA, 18:3n-3) found at around 10% (w/w) of the total fat content of canola oil and even higher in flaxseed oil. In general, however, humans do not have the enzymatic machinery to elongate and desaturate ALA to EPA by any more than 5-7% (Burdge and Calder, 2005; Goyens *et al.*, 2005; Harper *et al.*, 2005) due to linoleic acid (LA, 18:2n-6) competing with ALA at the level of the Δ 6-desaturase (see **Figure 4**).

Table 1. Common fatty acids arranged in difference classes							
Common name	Scientific name	Molecular	Abbrevi				
		name	ation				
Saturated fatty acids							
Lauric acid	Dodecanoic acid	12:0					
Myristic acid	Tetradecanoic acid	14:0					
Palmitic acid	Hexadecanoic acid	16:0					
Stearic acid	Octadecanoic acid	18:0					
Arachidic acid	Eicosanoic acid	20:0					
Behenic acid	Docosanoic acid	22:0					
Lignoceric acid	Tetracosanoic acid	24:0					
Monounsaturated fatty acids							
Vaccenic acid	11-octadecenoic acid	18:1 n-7					
Oleic acid	9-octadenoic acid	18:1 n-9					
Omega 3 polyunsaturated fatty acids							
α -linolenic acid	9,12,15-octadecatrienoic acid	18:1 n-3	ALA				
Eicosapentaenoic acid	5,8,11,14,17-eicosapentaenoic acid	20:5 n-3	EPA				
Docosapentaenoic acid	7,10,13,16,19-docosapentaenoic acid	22:5 n-3	DPA				
Docosahexaenoic acid	4,7,10,13,16,19-docosahexaenoic acid	22:6 n-3	DHA				
Omega 6 polyunsaturated fatty acids							
Linoleic acid	9,12-octadecadienoic acid	18:2 n-6	LA				
γ-linolenic acid	6,9,12-octadecatrienoic acid	18:3 n-6	GLA				
Arachidonic acid	5,8,11,14-eicosatetraenoic acid	20:4 n-6	AA				
n/a	4,7,10,13,16-docosapentaenoic acid	22:5 n-6					

(Footnote: all unsaturated FAs shown in this table are of the *cis* configuration.)



Figure 4. Diagrammatic representation of the n-6 and n-3 FA series metabolic pathway. The enzymes for each step are included in italics. AA and EPA are released from the membrane phospholipids by phospholipase A_2 and metabolized to the eicosanoids (see Figure 5).

1.6. Eicosanoid synthesis from membrane phospholipid pool.

Studies have shown that n-3 FAs (EPA and DHA) appear to be incorporated rapidly and preferentially into mammalian cell membrane phospholipid pools (Owen *et al.*, 2004; Patten *et al*; 2005a) compared with n-6 FAs. The resultant fatty acids released by the enzymic action of phospholipase A_2 from the membrane phospholipids, mainly from macrophages in the inflammatory response, are converted to the various eicosanoid classes by cyclooxygenases and lipoxygenases (see **Figure 5**). Generally, the 2-series prostaglandins and thromboxanes and 4-series leukotrienes such as PGE₂ and LTB₄ from n-6 FAs are considered proinflammatory while the 3-series prostaglandins and thromboxanes and 5-series leukotrienes such as PGE₃ and LTB₅ from n-3 FAs are regarded as less inflammatory. In industrialized



Figure 5. Diagram of eicosanoid synthesis from arachidonic acid and EPA. The main source of eicosanoids are macrophages and as a general indication, eicosanoids from the 2and 4-series are regarded as proinflammatory, whereas eicosanoids from the 3- and 5-series are regarded as less proinflammatory. Cyclooxygenase produces prostaglandins whereas lipoxygenase produces leukotrienes. Abbreviations for major classes: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; 5-HEPE, 5-hydroxyicosapentaenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HPETE, 5hydroperoxyeicosatetraenoic acid; LT, leukotriene; LTA₄, leukotriene A₄, 5-PEPE, 5peroxyeicosapentaenoic acid; PG, prostaglandin; PGI₂, prostaglandin I₂; TXA₂, thromboxane A₂. Adapted from Emprey et al., 1990 and Mills et al., 2005.

society the ratio of n-6:n-3 FAs is high due to increased consumption of n-6 rich vegetable oils

and decreased consumption of n-3 rich foods such as oily fish. Epidemiological studies

suggest that the human intake of n-6 FAs has increased the n-6:n-3 ratio from 1 to around 15 (Mills *et al.*, 2005). The full impact of this large shift in the n-6:n-3 ratio in our diet and subsequent FA makeup of our bodily tissues is the subject of much debate and clinical research. However, there are also concerns about the oxidative stress that can result from increases in dietary n-3 FAs if they are not accompanied by adequate amounts of vitamin E or other antioxidants present in higher levels in more primitive diets (Camuesco *et al.*, 2006).

1.7. Effects of n-3 PUFA on contractility of normal and ischaemic cardiac muscle

It is generally believed that dietary LC n-3 PUFA benefit cardiovascular disease outcomes. However, the results from large clinical trials (eg GISSI and DART [1 and 2] trials) (Marchioli *et al.*, 2002; Burr, 2007) and subsequent reviews (Hooper *et al.*, 2007) and meta-analysis (Wang *et al.*, 2006) have produced both positive and neutral outcomes for fish oil n-3 PUFAs. Studies with animals and isolated neonatal and adult cardiomyocytes using diets or media enriched with n-3 PUFA have investigated the biochemical mechanisms involved with this proposed cardioprotection (McLennan *et al.*, 1988, 1992a,b; Kang and Leaf 1995; Billman *et al.*, 1999; Jahangiri *et al.*, 2000; Leifert *et al.*, 2000a,b, 2001; Kukoba *et al.*, 2003).

The basis of this work commenced in the early seventies from the observations of Bang and Dyerberg concerning Greenland west coast Eskimos (Bang *et al.*, 1971) who, although consuming a high fat diet, were believed to have low rates of cardiovascular disease which was linked to their high intake of marine blubber and fat containing high amounts of LC n-3 PUFAs. The subsequent studies by Gudbjarnason, established that feeding fish oil lead to an incorporation of the LC PUFAs, EPA and

DHA into rat heart membrane phospholipid fractions at the expense of n-6 PUFA, LA and AA (Gudbjarnason and Hallgrimsson, 1976; Gudbjarnason and Oskarsdottir, 1977) and provided an explanation as to how such dietary changes related to human cardiovascular disease based on the differences in the n-6:n-3 FA ratios (Gudbjarnason *et al.*, 1989). However, McLennan *et al.*, (1988) were the first group to show that feeding tuna fish oil supplemented diets rich in n-3 FA to rats for several months prevented ischaemia-induced fatal ventricular arrhythmias which they subsequently confirmed in the marmoset monkey (McLennan *et al.*, 1992a,b). Others soon repeated these findings in the rat (Hock *et al.*, 1990). It appeared that for animal models in general, saturated animal fat was pro-arrhythmic while replacement with FAs of the n-6 class and, more particularly n-3 PUFA, but not monounsaturated fatty acids, could reduce the likelihood of an ischaemic event leading to sudden cardiac death (McLennan, 1993). The challenge was to establish the mechanisms involved in fish oil protection from normoxic and ischaemic-induced cardiac arrhythmias.

The possible mechanisms of n-3 PUFA action on heart rhythm may represent pleiotropic effects of such fats on several aspects of structure, metabolism, the autonomic nervous system and electrophysiology. Fish oil or pure n-3 fatty acid preparations have been efficacious in short and long term feeding trials in various animal models and upon acute addition to asynchronously beating isolated cardiomyocytes (see above). Fish oil LC n-3 PUFAs are incorporated into heart muscle tissue membranes at relatively higher concentrations over a period of several weeks (Owen *et al.*, 2004). A consequence of this increased n-3 PUFA incorporation may be effects on membrane fluidity (Leifert *et al.*, 2000a), mechanisms of cell signally including G-protein coupled receptors (Patten *et al.*, 1989), altered eicosanoid

production such as reduced thromboxane A_2 levels (Abeywardena *et al.*, 1991a,b; Bryan *et al.*, 2006; Moller and Lauridsen, 2006), and even effects on cardiac nuclear receptors and altered gene expression including protein kinase C regulation (Hlavackova *et al.*, 2007). Initial studies evaluating the global gene expression profile using array technologies in cultured neonatal rat cardiomyocytes supplemented with n-3 PUFAs detected upregulation of genes related to lipid transport. Many of the downregulated genes appeared to be related to inflammation, cell growth, extracellular and cardiac matrix remodelling, calcium movements and the generation of reactive oxygen species (ROS) (Bordoni *et al.*, 2007).

Studies using isolated cardiomyocytes have indicated direct modulation of Na⁺ currents and L-type Ca²⁺ channels (Leaf *et al.*, 1999; Leifert *et al.*, 1999; Leaf, 2001). Specifically, Leifert *et al.* (2001) demonstrated that in rats fed diets supplemented with fish oil, which in isolated heart cells in the presence of the sarcoplasmic reticulum Ca²⁺ pump inhibitor, DBHQ, the time constant of decay of Ca²⁺ transients (τ) induced by single electronic pulses was higher compared to a saturated fat control group. As mentioned, dietary fish oil feeding also leads to enhanced gene expression of several antioxidant enzymes involved in scavenging of ROS which could prevent reperfusion induced arrhythmias due to rises of $[Ca^{2+}]_i$ (Jahangiri *et al.*, 2006). Since calcium is integral to muscle contractility, n-3 PUFA mechanisms which control or limit $[Ca^{2+}]_i$ may ultimately play the key role in the prevention of fatal normoxic or ischaemic-induced ventricular fibrillation.

A critical factor in patients who have suffered a myocardial infarction is compromised left ventricular systolic dysfunction (Macchia *et al.*, 2005). What has been

demonstrated using appropriate animal models is that dietary n-3 PUFA positively affect the contraction of isolated rat papillary muscle in response to α -1 and β -adrenergic stimulation (Skuladottir and Johannsson, 1997), decrease the load dependence of relaxation (Chemla *et al.*, 1995), and increase the left ventricular ejection fraction in marmoset monkeys due to enhanced filling (McLennan *et al.*, 1992a,b). In the isolated working rat heart perfused with porcine blood at a haematocrit of 40%, a dietary fish oil group had reduced oxygen consumption at any given work output and increased post-ischaemic recovery compared to saturated fat or n-6 PUFA dietary supplemented animals (Pepe and McLennan, 2002).

In summary, dietary fish oil n-3 PUFAs act at the physiochemical and metabolic level and on gene expression increasing the antioxidant capacity of the heart, reducing endothelial cell damage, altering membrane fluidity affecting enzyme activities and stabilizing ion channels regulating $[Ca^{2+}]_i$ while reducing oxygen demand. Overall, these effects of n-3 PUFA improve the potential ability of the heart to function more efficiently, especially under conditions of stress.

1.8. Effects of n-3 PUFA on contractility of vascular smooth muscle cells and blood flow

Part of the cardiovascular benefits of n-3 PUFA noted in human and animal studies are likely to be mediated at the levels of the vascular endothelium (Mano *et al.*, 1995; Mori, 2006). This thin monolayer of cells plays a central role in cardiovascular homeostasis and function via the production of a range of potent autocrine and paracrine biochemical mediators (Abeywardena and Head, 2001) controlling the tone of vascular smooth muscle cells (VSMC) (Gibbons, 1997). Vasorelaxants include nitric oxide (NO), and endothelial derived hyperpolarizing factor (EDHF). Vasoconstrictors include angiotensin II, the potent endothelins, thromboxane A_2 /prostaglandin H_2 (PGH₂), prostaglandin $F_{2\alpha}$ (depending on the smooth muscle), superoxide anion and isoprostane (see **Figure 6**). The endothelium is also the site of many receptors, binding proteins, transporter and signalling processes involved in cell growth, apoptosis and cell migration. In certain disease states the endothelium may also produce increased levels of eicosanoids and free radicals and promote abnormal contraction of blood vessels. There is, therefore, a delicate interplay between the cells lining the vasculature and the VSMC whose tone regulates blood flow and blood pressure.

It has been postulated that LC n-3 PUFAs positively influence NO production and eicosanoid biosynthesis and hence vascular reactivity (Harris *et al*, 1997). Abeywardena *et al.* (1987) demonstrated that long term dietary supplementation of rats with different fats altered aortic PGI₂ and TXB₂ formation with tuna fish oil suppressing their production. A saturated fat group showed the highest PGI₂/TXB₂ ratio compared to a sunflower seed supplemented diet with tuna fish oil showing the lowest PGI₂/TXB₂ ratio. This may be partly explained by an increase in synthesis of PGI₃ and TXA₃. PGI₃ is equipotent to PGI₂ with regards to vasodilatory action whilst TXA₃ has little vasoconstricting activity (Abeywardena and Head, 2001). However, depending on the model and conditions, this hypothesis needs further testing (Hornstra *et al.*, 1981; Oudot *et al.*, 1998). Using adult human saphenous vein endothelial cells, Urquhart *et al.*, (2001), demonstrated that incubation for 72 hours with 50 μ M EPA or DHA inhibited basal production of the vasoconstrictor PGF₂ α by



Figure 6. The endothelium produces a range of vasoactive compounds involved with vascular smooth muscle homeostasis. Abbreviations: NO, nitric oxide; PGI2, prostaglandin I₂; EDHF, endothelial derived hyperpolarizing factor; AII, angiotensin II; ET, endothelin; PGF_{2α}, prostaglandin F_{2α}; TXA₂/PGH₂; thromboxane A₂/prostaglandin H₂; O₂⁻, superoxide anion. Modified from Abeywardena and Head, 2000.

50% and 80%, respectively. It has also recently been reported that cytochrome p-450 epoxygenase metabolites of DHA can potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels (Ye *et al.*, 2002). These and other such interactions may form the basis for the reported improvement by n-3 PUFA of endothelial function and arterial elasticity possibly via ion channel activation (Asano *et al.*, 1997, 1998; Goodfellow *et al.*, 2000; Nestel, 2000; Conde *et al.*, 2007) that ultimately leads to reduction of blood pressure described in animal models (Head

et al, 1991; Mano *et al*, 1995). A list of proinflammatory cytokines, or cytokines reflecting inflammatory processes that are reduced by ingestion of EPA and DHA would include the following: IL-1 β , IL-2, IL-6, TNF α , and platelet derived growth factor (PDGF)-A.

The effects of fish oil on atherosclerosis act in part via inhibition of VSMC proliferation and migration, modification of expression of COX-2 and inflammatory cytokinesis (see above) and adhesion molecules (VCAM-1, ICAM-1 and E-selectin), reduction of oxidative stress (as indicated by 8-iso-PGF_{2 α}), and the stabilization of plaque formation (De Caterina et al., 1994, Shimizu et al., 2001; 2004; Chen et al., 2005; Machida et al., 2005; von Shacky, 2007a,b) (see Figure 7 and Figure 8 for fish oil effects on blood vessels). In an earlier study, the endotoxemic rat model demonstrated reduced rates of blood flow studied using radioactive microspheres to the small and large intestines, stomach, skin and skeletal muscle (Pscheidl et al., 1992). Short term intravenous feeding with n-3 PUFAs has been reported to increase portal and intestinal blood flow. This occurred with a concomitant improvement in glucose tolerance (Pscheidl et al., 1992). Enteral feeding containing fish oil also increased blood flow to the ileum of the rat. Concomitant with these effects was an altered expression of the proinflammatory cytokines, IL-4 (increased) and IL-10 (decreased) (Matheson et al., 2003). In streptozocin-induced diabetes in the rat, n-3 PUFA supplementation with Promega (0.5 mL/kg/d) for 4 weeks beginning 2 weeks after diabetes induction had negligible effects on the levels of plasma glucose, triglyceride (TG) or cholesterol. However, both aortic and coronary blood flow rates were increased in diabetic rats fed n-3 PUFA in a working heart model where membrane n-3 FA levels were increased (Balck et al., 1993). In vitro studies



Figure 7. Summary of the proposed beneficial effects of fish oil LC n-3 PUFA (EPA and DHA) on blood vessel function. A more comprehensive list of n-3 FA effects on blood vessels and other selected tissues is shown in **Figure 8** and **9**. Adapted from Abeywardena and head, 2000.

employing aortic rings from rats fed 20% fat diets (w/w) showed that under preanoxic or post-anoxic conditions, rings from rats fed fish oil and corn oil enriched diets contracted less than rings from rats fed diets enriched with saturated beef tallow. The relaxation response to acetylcholine, however, was greater in aortic rings from rats fed diets supplemented with fish oil. As a consequence, this may result in increased blood flow to ischaemic and reperfused tissues *in vivo* (Malis *et al.*, 1991). High fish oil supplementation also led to increased reperfusion blood flow after 40 min of left coronary artery occlusion of rat heart with no effect on the extent of myocardial infarction area (Force *et al.*, 1989).

In a later study, healthy humans were supplemented with a high n-3 PUFA diet of DHA (2 g/day) and EPA (3 g/day) or a sunflower (5 g/day) control for 6 weeks. The n-3 PUFA supplemented group had enhanced brachial artery blood flow and conductance during a hand grip exercise (Wasler *et al.*, 2006). It has also been

NOTE: This figure is included on page 32 of the print copy of the thesis held in the University of Adelaide Library.

Figure 8. Possible mechanisms of endothelium-independent effects of n-**3 PUFA on lowering [Ca²⁺]***i* dynamics. Studies in experimental animal models and human subjects with hypertension demonstrate modulation of vascular tone and a modest reduction of BP after fish oil or n-3 PUFA dietary supplementation possibly by endothelium-dependent and –independent mechanisms. Abbreviations: DHA docosahexaenoic acid; DPA, docosapentaenoic acid (n-3); EPA, eicosapentaenoic acid; *I*_{cat}, non-selective cation channel; PKC, protein kinase C; SR, sarcoplasmic reticulum, VSMC, vascular smooth muscle cell. Modified from Hirafuji *et al.*, 2003.

demonstrated that n-3 PUFA enhances sympathetic nerve activity during forearm

contractions (Monahan et al., 2004).

To examine these phenomena, isolated smooth muscle cells in the form of passaged foetal rat aortas (A7r5 cells) were incubated for up to 7 days with media supplemented with 30 µM n-3 PUFA and the resulting effects on ions currents and ion handling were examined using the whole cell voltage clamping technique and the Ca²⁺-sensitive dye fura-2 AM (Asano et al., 1997; 1998). After treating the A7r5 cells with EPA, the EPA and docosapentaenoic acid (DPA, 22:5n-3) content of the cellular phospholipid fraction increased in a time dependent manner. Alternatively, AA decreased and then the ratio of EPA and AA (EPA/AA) increased significantly (Asano et al, 1998). In the first study, the major findings were 1) n-3 PUFA (EPA and DHA) induced a K⁺ current in rat A7r5 smooth muscle cells; 2) EPA, DHA and DPA at concentrations of 3-100 µM inhibited the receptor-mediated non-selective cation current (I_{cat}) activated by the vasoconstrictors vasopressin and endothelin-1 (ET₁). 3) These findings indicated that n-3 FAs play an important role in the control of vascular tone while the site of action of the n-3 FAs did not involve the peptide hormone receptors for vasopressin and endothelin-1 as described above (Asano et al., 1997). In a following experiment, A7r5 cells were similarly incubated with media supplemented with EPA. The resting $[Ca^{2+}]_i$ was significantly decreased from 170 nM in control treated (oleic and stearic acid) cells down to 123 nM in cells treated with EPA supplementation in the media. Vasopressin and ET₁ (both 100 nM) and plateletderived growth factor (PDGF, 5 ng/mL) evoked an initial peak of $[Ca^{2+}]_i$ followed by a smaller sustained rise of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} . In EPA-treated A7r5 cells, both the peak and the sustained rise in $[Ca^{2+}]_i$ induced by agonists decreased significantly in comparison to control cells. The resting membrane potential was also significantly higher in EPA-treated A7r5 cells than in control treated cells (Asano et al., 1998). Combined, these results from cultured VSMC from the large

NOTE: This figure is included on page 34 of the print copy of the thesis held in the University of Adelaide Library.

Figure 9. Possible mechanisms for anti-proliferative and pro-apoptotic effects of n-3 PUFAs from marine oils as mediators of anti-atherogenic effects on VSMCs. EPA and DHA have been reported to regulate VSMC proliferation/migration induced by several mitogens. In particular DHA can cause apoptosis via multiple mechanisms involving nuclear receptors and gene expression. Indirect autocrine effects via the modulation of PGI₂/PGI₃ and NO production may contribute to the antiatherogenic mechanism. Abbreviations: cdk2, cyclin-dependent kinase-2; 5-HT₂, serotonin-2; $\Delta\Psi$ m, mitochondrial transmembrane potential; PDGF, platelet derived growth factor; PKC, protein kinase C; PS, phosphatidyl-serine; PPAR-α, peroxisome proliferator-activated receptor-α; NO, nitric oxide; TGF-β; transforming growth factor-β; TXA₂, thromboxane A₂. Adapted from Hirafuji *et al.*, 2003.

conductance vessel help explain the effects of n-3 PUFA on blood vessel contractility in terms of altered calcium homeostasis and its concomitant effects on the contractile cycle.

EPA treatment has also been shown to inhibit platelet-derived growth factor (PDGF)induced rodent and human vascular cell migration thus contributing to the antiatherosclerotic effects described for n-3 PUFA (Mizutani *et al.*, 1997). Short term EPA treatment (24 h) also suppresses VSMC migration induced by oxidized LDL and lyso-PC (Kohno *et al.*, 2000). It is clear that n-3 PUFA biochemistry in healthy and diseased states is complicated due to the interplay between the endothelium and contracting VSMC that results in altered blood flow that is critical to compromised ischaemic tissue, be it coronary vessels of heart, brain circulation, or blood vessels of gut, retina, or to peripheral tissue affected by diabetic neuropathy. It could be concluded that LC n-3 PUFA supplementation and subsequent membrane incorporation leads to healthier blood vessel endothelium via ionic modulation and hence improved vascular reactivity and blood flow when tissue is stressed (see **Figure 8** for beneficial effects of n-3 PUFA on blood vessels and for more specific antiproliferative and pro-apoptotic effects on blood vessels, see **Figure 9**).

1.9. Effects of n-3 PUFA on contractility of gastrointestinal tissue

Unlike cardiac and vascular tissue, there is a paucity of information on the role of n-3 PUFAs on gastrointestinal contractility and motility (Jonkers *et al.*, 2000). Only recently have the connections between n-3 PUFA modulation of contractility and the potential for protection and/or amelioration from gut inflammatory conditions been evaluated (Belluzzi, 2004; Cao *et al.*, 2005; Al-Jarallah *et al.*, 2007). However,

contractility studies have not been carried out in the past with dietary n-3 fatty acid (and other) interventions in normal animal models or where the contractility of the gastrointestinal tract has been compromised.

Nevertheless, it has been reported that the acute administration of LC PUFA from fish oil into the duodenum of healthy humans leads to significantly shorter gallbladder contraction duration compared to corn oil with both fats inducing a postprandial antroduodenal motility pattern while not influencing small bowel transit time (Jonkers et al., 2003). Both LC and medium chain fatty acids decrease lower oesophageal sphincter pressure (Ledeboer et al., 1998). The satiety factor, cholecystokinin (CCK), has a lower rate of secretion after fish oil infusion compared to corn oil, while other gastrointestinal hormones, peptide YY (PYY) and neurotensin release were not influenced. It appears that the effects of fatty acids on CCK release and gall bladder motility are dependent on fatty acid chain length. Hypertriglyceridaemia is a not only a risk factor for CVD, but also for gall stone formation due to saturation with cholesterol and subsequent gall bladder dysmotility as a result of a decreased sensitivity to CCK. A recent study has demonstrated that triglyceride (TG) lowering therapy by a bezafibrate or a high fish oil dietary supplementation (5 g/day) improved gall bladder dysmotility without adversely affecting biliary cholesterol saturation (Jonkers et al., 2003) although it is generally thought that n-3 PUFA have a more significant effect on triacylglycerides than cholesterol. The mechanism is possibly hypertriglyceridemia-induced lipid perturbation of the smooth muscle membrane bilayer, altering the interaction of CCK with its receptor and/or the CCK receptor-G protein interaction and downstream Ca²⁺ modulation leading to an altered physiological response.
1.10. Definition of fibre and resistant starch

Since 1953, when nutritionist EH Hipsley first coined the term "dietary fibre", the scientific community have acknowledged its nutritional potential (Hipsley 1953; Cummings, 1978). According to the National Academy of Sciences (2002) the definition is condensed to the following: "Dietary Fibre consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants. Functional Fibre consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans. Total Fibre is the sum of Dietary Fiber and Functional Fiber." There are also three main categories of dietary fibre; soluble, insoluble and resistant starch, with each demonstrating specific health benefits. The key point is that dietary fibre survives passage through the small intestine and is attacked by enzymes of colonic microflora yielding short chain fatty acids (SCFAs), hydrogen, carbon dioxide and methane as fermentation products (Escudero and Gonzalez, 2006). The main SCFAs are acetate, propionate and butyrate accounting for 90-95% of the total colonic SCFA pool (Kamath and Phillips, 1988) with SCFAs reaching concentrations of 60-130 mM in the proximal colon of humans (Cummings et al., 1987). SCFAs may affect blood glucose and lipid levels (Higgins, 2004), improve the colonic environment by maintaining a lower pH (which limits the production of carcinogens), regulating the immune responses (Harris and Ferguson, 1993) as well as being an important energy source for colonocytes and possibly the colonic bacteria themselves (Rose et al., 2007). However, the degree to which these fibre types generate SCFAs (and butyrate in particular) is still unclear. In the context of this literature review, discussion will also cover the influence of SCFA on aspects of gastrointestinal motility (Cherbut et al., 1996; Dass et al., 2007).

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1.11 Effects of dietary fibre on contractility of the gastrointestinal tract

Dietary fibre is thought to enhance colonic transit and frequency by increased faecal bulk and to affect smooth muscle contractility via the production of SCFAs. Low-fermentable fibre more effectively increases faecal bulk rather than fermentable fibre because of its water-retaining capacity. SCFAs on the other hand, which may also be produced from undigested protein (MacFarlane and MacFarlane, 2003), can modulate neural and hormonal pathways (Mitsui *et al.*, 2006). Physiological studies have implicated SCFAs as a luminal chemical stimulus which can control gastrointestinal contractility and motility at the level of the rumen (Kendall and McLeay, 1996) stomach (Cuche and Malbert, 1999a), small intestine (Cuche and Malbert, 1999b; McManus *et al.*, 2002) and colonic longitudinal muscle (Cherbut *et al.*, 1998). However, whether the effects of SCFAs are stimulatory or inhibitory is unclear, and varies according to the different experimental paradigms (Yajima, 1984; Masliah *et al.*, 1992; Squires *et al.*, 1992; Fukumoto *et al.*, 2003).

Bulking action causes firing of stretch-sensitive enteric nervous tissue probably via serotonin (5-HT) release whilst SCFAs may elicit contraction of gut tissue via an anionic hyperpolarisation effect or at the newly discovered G-protein coupled receptors GPR41 and GPR43 (Fukamoto *et al.*, 2003; Nilsson *et al.*, 2003). At present, these receptors can only be distinguished by their rank order potency to SCFAs. Compared with propionate and butyrate, acetate has lower potency at GPR41 receptor and is equipotent at the GPR43 (Brown *et al.*, 2003). However, SCFA-induced contractions can occur independently of the GPR43 receptor (Dass *et al.*, 2007) which, in contrast are only poorly expressed in the small intestine (Le Poul *et al.*, 2003) or colonic muscle tissue (Karaki *et al.*, 2007) compared to the spleen and

polymorphonuclear cells. However, the GPR43 receptor is expressed at higher levels both in intestinal enterochromaffin cells and mucosal mast cells (Karaki *et al.*, 2006) which express PYY and 5-HT, and as mentioned, in specific types of white blood cells (Nilsson *et al.*, 2003). It is thus possible that SCFAs influences gut smooth muscle cell contractility via a paracrine route. This proposition is supported by the recent finding that SCFAs at physiological concentrations may accelerate colonic transit by increasing the release of 5-HT and calcitonin gene-related peptide from mucosal cells when applied to flat-sheet preparations of the rat middle to distal colon (Grider and Piland, 2007).

A recent study has noted that feeding rats a fibre-free diet resulted in a significantly lower colonic weight while the thickness of muscle layer and total body weight was not significantly affected (Mitsui *et al.*, 2006). When myogenic responses of circular muscle were measured in the presence of tetrodotoxin (TTX binds to the pores of the voltage-gated, fast sodium channels thus isolating the myogenic effects), activation of muscarinic receptors by carbachol resulted in significantly greater contractions in a group supplemented with dietary cellulose compared to a fibre-free group. There were no reported changes in sensitivity (EC₅₀) in response to carbachol stimulation on the longitudinal smooth muscle. When contractions induced by substance P were examined in the presence of TTX and the muscarinic acetylcholine receptor antagonist, atropine, there were no differences in the substance P-induced contractions of circular muscle strips of distal colon with regard to fibre in the diet. However, in the longitudinal muscle strips, substance P-induced contractions of the fibre-free group were significantly larger compared to the fibre-fed groups (Mitsui *et al.*, 2006). The fibre-free diet led to a decrease in the colon enterochromaffin cell number. It is important to note that enterochromaffin cells are involved with the release of the neurotransmitter 5-HT when the colon is mechanically stimulated by faeces (Wade *et al.*, 1996). It was concluded that functional changes of enteric neurons and muscle cells, as well as a decrease in the number of EC cells, could affect the colonic motility of rats fed fibre-free diets.

1.12. Conclusions

The mechanisms of action of dietary fish oil and fibre in animal physiology are complex and are not fully understood. The n-3 PUFAs are readily incorporated into tissue membranes in a preferential manner at the expense of n-6 PUFAs such as linoleic acid (18:2 n-6) or arachidonic acid (20:4n-6). In general, studies have demonstrated that when n-3 FAs (EPA and DHA) are incorporated into the phospholipid membrane of cells at the expense of AA, the resultant eicosanoids released in the inflammatory cascade, including PGE₃, are less proinflammatory than the prostaglandins classes 1 and 2, thromboxanes A₂ and leukotrienes (especially LTB₄, see **Figure 4**) (Fritsche *et al.*, 1999; Calder, 2003; Mills *et al.*, 2005). The LC n-3 PUFA also act as ligands of PPAR α involved in fat oxidation (Price *et al.*, 2000) and affect the transcription of several genes involved in the control of inflammation and metabolism.

In addition n-3 PUFAs are incorporated into plasma membrane lipids, where they modify membrane physicochemical properties such as fluidity and influence enzyme activity, ion channels and receptor system binding. This results in a decrease in vascular smooth muscle tone which leads to lower BP and improved blood flow (Pscheidl *et al.*, 1992; Abeywardena and Head, 2000). On the other hand, in gut

smooth muscle and cardiac tissue n-3 PUFAs lead to increased contractility (McLennan *et al.*, 1993; Patten *et al.*, 2002a, 2005a,b, 2006). These outcomes are probably due to modification of Ca²⁺ handling mechanisms, eicosanoid production and endothelial derived relaxing factors such as NO. The newly discovered class of mediators, resolvins, lipoxins and docosatrienes (Arita *et al.*, 2005), may help explain some of the biochemical mechanisms involved in LC n-3 PUFA protection in inflammatory conditions. For a summary of general effects of dietary LC PUFA from fish oil on selected tissues including platelets, blood vessels, cardiac tissue and gut, refer to **Figure 10**.

Fibre, and in particular, resistant starch, reaches the large bowel where bacterial fermentation produces SCFAs, the major products being acetate, propionate and butyrate which in combination maintain bowel pH at healthy levels. As well as increased faecal bulk and laxation, dietary fibre assists with the secretion of carcinogens in the faeces, decreases resorption of bile salts, decreases conversion of primary bile acids to secondary bile acids and decreases the rate of glucose absorption in the small intestine. Furthermore, dietary fibre is important for development of colonic enterochromaffin cells which are involved with the paracrine modulation of bowel contractility. Indeed, SCFA have been implicated in the modulation of contractility along the majority of the gastrointestinal tract from stomach to large bowel, but the mechanisms of such actions have yet to be fully elucidated. Butyrate, in particular, is a key substrate for the cells lining the gut wall and this four carbon fatty acid is implicated in the regulation of cell proliferation and apoptosis and the prevention of bowel cancers. Specifically, the physiological responses to butyrate are

Effects of fish oil LC n-3 PUFA on selected tissues			
Platelets DHA & EPA↑ Fluidity (DHA)↑ Aggregation↓ TNFα↓ PDGF-A & -B↓ PI-PKC signal↓	Heart DHA & EPA↑ Arrhythmia↓ Ejection fraction↑ Voltage dependent L-type Ca ²⁺ channels↓ Na ⁺ current↓ Sarcolemmal Ca ²⁺ efflux↓ IP ₃ ↓ SR (Ca ²⁺ -Mg ²⁺)- ATPase↓ TBX ₂ ↓	$\begin{array}{c} \underline{\text{Blood vessels}}\\ \overline{\text{DHA \& EPA}}\\ \overline{\text{BP}}\downarrow\\ \overline{\text{VSMC}}\downarrow\\ \overline{\text{PDGF-induced}}\\ \overline{\text{VSMC migration}}\downarrow\\ \overline{\text{Tone}}\downarrow\\ \overline{\text{Tone}}\downarrow\\ \overline{\text{Blood flow}}\uparrow\\ \overline{\text{PKC}}\downarrow\\ \overline{\text{[Ca}^{2+}]_i\downarrow}\\ \overline{\text{Ca}^{2+}} \text{ channels}\downarrow\\ \overline{\text{K}^+(\text{ATP}) \text{ channel}}\uparrow\\ \overline{\text{Non-selective }I_{cat}}\downarrow\\ \overline{\text{K}^+(\text{ATP}) \text{ channel}}\uparrow\\ \overline{\text{Non-selective }I_{cat}}\downarrow\\ \overline{\text{INOS}}\uparrow\\ \overline{\text{TBX}_2\downarrow}\\ \overline{\text{TNF}_{\alpha}}\downarrow\\ \overline{\text{IL-1}_{\beta}\downarrow}\\ \overline{\text{LTB}}\downarrow\\ \overline{\text{VEGF}}\downarrow\\ \overline{\text{IL-1}_{\beta} \text{ COX }2i}\\ \overline{\text{mRNA}}\downarrow\\ \overline{\text{PGE}_2\downarrow}\\ \overline{\text{P44}/42 \text{ MAPK}}\downarrow\\ \overline{\text{Ach relaxation}}\uparrow\\ \end{array}$	GutDHA & EPA^Agonist-inducedcontractility^Electrically-drivencontractility^ M_1 receptors^Blood flow^Inflammation \downarrow IL-1 $\beta \downarrow$ IL-1 $0 \downarrow$ IL-1 $0 \downarrow$ IL-1 $5 \downarrow$ TBX ₄ \downarrow TNF- $\alpha \downarrow$

Figure 10. Summary of general effects of dietary LC PUFA from fish oil on selected tissues including platelets, blood vessels, cardiac tissue and gut. The table summarizes many of the points discussed in the text or from the references therein. Abbreviations: Ach, acetylcholine; ATPase, adenosine triphosphatase; BP, blood pressure; COX 2i, cyclooxygenase 2 (inducible); DHA; docosahexaenoic acid; EPA, eicosapentaenoic acid; IL-1_β, interleukin 1_β; IP₃, inositol triphosphate; LC, long chain; LTB, leukotriene B; M₁, muscarinic subtype 1; mRNA, messenger ribose nucleic acid; iNOS, nitric oxidase synthetase (inducible); PDGF-A, platelet-derived growth factor A; PGE₂, prostaglandin E₂; P44/42 MAPK, P44/42 p38 mitogenactivated protein kinase; PI-PKC, phosphatidyl inositol-protein kinase C; SR, sarcoplasmic reticulum; TBX₂, thromboxane X₂; TNF_α, tumour necrosis factor-alpha; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.

a decreased production of proinflammatory cytokines, inhibition of nuclear factor-kB

activation and enhanced production of peroxisome proliferator-activated receptors,

which all result in a decreased inflammatory response. The role of butyrate as a

cognate ligand for the recently discovered G-protein receptor system (GPR43) is novel but needs to be fully defined (Karaki *et al.*, 2007).

What is becoming evident is that dietary fibre and in particular n-3 PUFA is positively indicated in a multitude of ways for good health, in a balance that provided well for our ancestors in the preceding thousands of years (2002a,b). The evidence for fish oil for CVD protection is extensive and is growing for cancer prevention, but even so, these concepts are still controversial (Hooper *et al.*, 2007). The challenge is to substantiate the role of dietary LC n-3 PUFA and fibre on indices of gastrointestinal health (Turner *et al.*, 2007a,b).

Docosahexaenoic acid (DHA, 22:6n-3)



Experimental studies related to this thesis

2.1. Methodology

Investigative studies of isolated gut tissue contractility was established in organ bath by modification of previous methods (Paton and Visi, 1969; Brantl et al., 1979) for the simultaneous bathing of the serosa of an intact piece of isolated guinea pig ileum while allowing infusion of the isolated lumen (Patten et al., 2001). Electrical stimulation was introduced via parallel stainless steel rods. In the rat, an open system of superfusion was developed for ileum and colon with opposite circular stainless steel leads initiating electrically-driven contraction of the colon (Patten et al., 2005b). Various gastrointestinal agonists of contraction (acetylcholine, histamine, serotonin, PGE_2 , $PGF_{2\alpha}$, and 8-iso- PGE_2) and inhibitors of electrically-driven contraction (morphine and epinephrine) could be introduced into the organ bath. The comparative compartmental potency of dietary-derived opioid blockers affecting morphine could be tested using guinea pig ileum. Finally, the effects of dietary intervention of various fats and fibre on lower gut contractility in healthy guinea pigs and rats or hypertensive rats that exhibited compromised prostanoid-driven gut contractility could be examined. It is of note that the rat intestinal system was not responsive to histamine or serotonin; they are both powerful neuroeffectors with their roles in diseases of the bowel yet to be defined. Interestingly, as demonstrated by others, the powerful eicosanoid aortic vasoconstrictor, 8-iso-PGF_{2 α}, showed little activity in isolated guinea pig or rat gut tissue (Sametz et al., 2000). However, the thromboxane A₂ mimetic, 9,11-dideoxy-9,11-methanoepoxy prostaglandin $F_{2\alpha}$ (U-46619), which is of relevance to inflammatory conditions of the gut was found to be a powerful stimulant of contraction of the rat ileum but not the colon. Of particular interest were the effects of the long chain (LC) n-3 polyunsaturated fatty acids found in high concentration in

fish oil on gut smooth muscle contractility because of their known effects on vascular smooth muscle and cardiac tissue contractility under normoxic conditions and regimens of ischaemic challenge.

Initial experiments using the guinea pig indicated that custom synthesized opioid antagonists casoxin 4 (a tetra-peptide sequence found in milk κ -casein) and its analogue [D-Ala²]-casoxin 4 when infused into the guinea pig lumen, significantly antagonised the inhibitory effects of morphine when added to the serosal side (Patten *et al.*, 2001). D-Alanine was substituted in the second position of casoxin to potentially minimize the action of peptidases (Read *et al.*, 1990). These effects may have implications for the treatment of opioid induced constipation (Paulson *et al.*, 2005).

2.2. Dietary fibre feeding trial in newborn guinea pigs

In the first dietary study using the guinea pig model, convenience rice congee (a staple Asian food) supplemented diets were tested against other equal content fibre sources for eight weeks for effects on young guinea pig gut growth, caecal SCFA levels and ileal contractility (Patten *et al.*, 2004b). While total caecal SCFA content did not significantly vary, butyrate was higher in a pulse based supplemented diet of baked beans. Contractility studies revealed a small but significantly higher voltage was required to initiate ileal contraction in a congee fed group compared to control dietary fibre groups which included baked beans. This may involve some intrinsic property differences between the gut tissues with regard to release of acetylcholine or other mediators such as serotonin or ionic handling properties of the smooth muscle membrane involved with depolarization and contraction between the animal groups.

This is difficult to explain, but may be a result of different natural dietary fibre or possible subtle differences in membrane fatty acid composition as a result of the dietary fat treatments. It may also involve the population of enterochromaffin cells whose density in gut tissue has been shown to be modulated by dietary fibre. Enterochromaffin cells release serotonin when mechanically stimulated (Wade *et al.*, 1996) and may act in an autocrine manner.

There were no significant changes however, in response to a wide range of GIT stimulators or inhibitors of contraction in the guinea pig. These included the major parasympathetic effectors in the myenteric plexus of smooth muscle, i.e. acetylcholine (Visi, 1973) and histamine, which are strong drivers of ileal contraction and are implicated in intestinal secretion (Sjoqvist *et al.*, 1992), vomiting (Fozard, 1987), bowel inflammation and disease (Gui, 1998), and normal motility (Nagakurra *et al.*, 2000). Also tested were prostaglandins which play an important role in the maintenance of human gastric mucosal homeostasis and repair (Reilly *et al.*, 1998), mucous secretion (McQueen *et al.*, 1983), blood vessel permeability and smooth muscle tone of isolated jejunum in animal models (Mohajer and Ma, 2000).

2.3. Fish oil feeding trial in newborn guinea pigs

For the next dietary trial, congee based diets supplemented with 3% (w/w) safflower oil or 3% high DHA tuna fish oil of the diet complemented with fruit and vegetables (approximately 50:50 of total weight) were fed to young guinea pigs for two months (Patten *et al.*, 2002b). This equated to approximately 1.5% dietary fat with fish oil feeding increasing levels of ALA, EPA and DHA (which as a total approximated 10% of the total membrane phospholipid fatty acid pool). This resulted in lower oleic acid

proportion in the ileal membrane total phospholipid fraction compared to safflower oil supplemented animals. Significantly less voltage was required to initiate contraction of the ileum of the fish oil supplemented group compared to the control group. Although the differences were small, this could also indicate some intrinsic property differences between the gut tissues with regard to release of acetylcholine or the ionic handling properties of the smooth muscle membranes leading to depolarization and contraction between the animals fed the two dietary fats. Addition of n-3 PUFAs has been found to influence ion currents and contractility of other smooth muscle types (Pehowich, 1998) and isolated cardiomyocytes (Leifert *et al.*, 1999, 2001: Leaf, 2007). However, it is most likely that the mechanisms associated with the acute addition of fatty acids (as free acids or methyl esters) to cell media are different from those evident following feeding studies where n-3 PUFAs have been incorporated into the membrane at the expense of mainly n-6 PUFAs.

In the study described above, there was no alteration in sensitivities of maximal contractile responses to acetylcholine, histamine, serotonin and the prostanoids, PGE₂ and PGF_{2a} that are key regulators of various gastrointestinal functions (Patten *et al.*, 2002b). However, there was an almost five fold decrease in sensitivity to the isoprostane, 8-*iso*-PGE₂ without a change in the maximal contraction. 8-*iso*-PGE₂ is an isoprostane formed by free radical mediated peroxidation of arachidonic acid (AA). The isoprostanes which exhibit potent biological activity (Morrow *et al.*, 1999) have been monitored as an indication of oxidative stress (Reilly *et al.*, 1998) and in association with some diseases including diabetes (Mori *et al.*, 1999b). In could be concluded from the first two dietary intervention studies with guinea pigs described above, that dietary fibre, and in particular, fish oil can influence contractility, albeit

subtlety, of isolated gut smooth muscle tissue under conditions of electrical stimulation or a gastrointestinal isoprostane modulator of contractility that may be involved in disease states. Compared to other n-3 PUFA dietary interventions, the final level of fish oil supplementation in the guinea pig experiment was only about 1.5% of the total diet mass. However, it was important to validate and extend these findings of n-3 PUFAs from fish oil and fibre on contractility in other animal models, such as the rat.

2.4. Fish oil feeding trial in Sprague-Dawley rats

Subsequently, in the first study using 9 week old Sprague-Dawley (SD) rats, the animals were fed 17% fat diets (w/w) of the total diet as Sunola oil (high oleic acid



Figure 11. Gastrointestinal tract of normal male Sprague-Dawley (SD) rat. The dissection shows tissue from the stomach (top) to the rectum (bottom). Contractility studies for guinea pig and rat used sections of small intestine called the terminal ileum approximately 5 cm proximal from the caecum and sections of proximal or distal colon as described. Scale is in centimetres.

content), or substituted with 10% saturated animal fat (beef and mutton dripping) or fish oil (high EPA) for four weeks (Patten et al., 2002a). Fish oil supplementation led to increased maximal contractility of around 100% in the ileum (see Figure 11 for the anatomical display of the rat GI tract) in response to acetylcholine and several eicosanoids involved in bowel health and disease including, PGE_2 , $PGF_{2\alpha}$, U-46619 and 8-iso-PGE₂ with no changes in sensitivity. It should be pointed out, that unlike the guinea pig, the ileum of rats was unresponsive to histamine and serotonin. For the SD rat itself, only the ileum and not the colon was responsive to the thromboxane mimetic, U-46619. This emphasizes the differences in the animal model chosen for experimentation. Importantly however, the changes in contractility were correlated with an increase in n-3 FA content of gut membranes. Interestingly, the colon which had a similar increase in n-3 PUFA profile did not have a significantly increased contraction as a result of dietary fish oil. It was concluded that the non-responsiveness in the colon was probably due to a limited physiologic role in a healthy state (Patten et al., 2002a). This expanded the findings in guinea pig and demonstrated for the first time that n-3 PUFA derived from a fish oil supplemented diet could alter the potential strength of contraction of gut smooth muscle tissue. What role LC n-3 PUFAs play in conditions where gut contractility is compromised was yet to be fully elucidated and is of interest to experimenters (Bassaganya-Riera and Hontecillas, 2006) and clinicians alike (Bjorkkjaer et al., 2006; Wild et al., 2007).

2.5. Dietary saturated fat feeding trial in WKY rats and SHR

Another important question was whether high levels of saturated fat (SF) known to influence lipoprotein metabolism (Han *et al.*, 2002) and the development of atherosclerosis (Christen, 2003) could alter gut contractility of a normal rat (WKY) or

the spontaneously hypertensive rat (SHR) model. Vascular smooth muscle function of SHR is known to be compromised and is over-reactive to various biological stimuli (Abeywardena and Head, 2001). Dietary SF and PUFA exert their pleiotropic physiologic actions by altering membrane fatty acid composition; this can modify mediator profiles such as eicosanoids and also affect physiologic responses to exogenous agonists. Therefore, in the next study rats were fed diets for 12 weeks containing 3% fat (w/w of total diet) as sunflower oil or supplemented with a further 7% or 27% lard to give 10% and 30% total fat, respectively (w/w of total diet) (Patten et al., 2004a). Lard was chosen as the source of fat because, although only 36% SF, the sn-2 position of the triacylglycerol contains 71% as SFA (mainly as palmitic acid, 16:0) which has been reported to influence the total phospholipid FA profile (Mu and Hoy, 2004). This wide range of dietary SF had no effect on the contractility responses of the ileum. On the other hand, in the colon there were subtle changes in sensitivity to angiotensin II in the WKY rat and a change of sensitivity to PGE₂ and carbachol in the SHR. However, when the results of the three dietary groups were combined, there was lower sensitivity and lower maximal contraction in ileum and lower maximal contraction in the colon of SHR in response to $PGF_{2\alpha}$ and PGE_2 compared with the WKY group. PGs are important for GI homeostasis, including mucous secretion and smooth muscle tone (Ferreira et al., 1972; McQueen et al., 1983; Eberhart and DuBois, 1995). In chronic inflammatory bowel diseases (IBD), e.g., Crohn's disease and ulcerative colitis (UC), the overproduction of various PGs has been reported (Subbaramaia et al., 2004). This was the first report of a defect in PG responsiveness from gut tissue from hypertensive rats (Patten et al., 2004a). In may be concluded that although patients with IBD rarely have hypertension (Pizzi et al., 2006), this

prostanoid defect in SHR gut may be a useful model for investigating bowel disease, especially where contractility is compromised.

It had been established that dietary fish oil rich in n-3 PUFA modulates gut contractility in the SD rat strain (Patten *et al.*, 2002a). It was further demonstrated that the gut of SHR had a depressed contractility response to PGs compared to normotensive WKY rats (Patten *et al.*, 2004a). The next important question to investigate was whether feeding diets supplemented with fish oil rich in n-3 PUFA could increase gut contractility in healthy rats and restore the depressed prostanoid response in the SHR.

2.6. Fish oil, saturated fat and canola oil dietary feeding trial in WKY rats and SHR

To answer the issues raised above, relatively large groups (n=16) thirteen-week-old SHR were fed diets containing 5g/100g (~5%) as coconut oil, lard, or canola oil containing 10% (w/w) n-3 FA as α -linolenic acid (ALA; 18:3n-3), or fish oil (as HiDHA[®], 22:6n-3) and a WKY control group fed coconut oil (Patten GS *et al.*, 2005b). In the first instance, this experiment indeed confirmed that the tissues of the coconut oil supplemented SHR group were less responsive to PGE₂ and PGF_{2 α} compared to tissues from the WKY coconut supplemented group. Feeding diets supplemented with fish oil to SHR increased the maximal contraction response to acetylcholine in the ileum compared to all diets confirming previous findings in a normotensive rat model (Patten *et al.*, 2002a). Fish oil also restored the depressed response to PGE₂ and PGF_{2 α} in the ileum but not the colon of SHR (Patten *et al.*, 2005b). Fish oil dietary supplementation again led to a significant increase in gut total

phospholipid n-3 PUFAs mainly as DHA, with lowered proportions of n-6 PUFA as arachidonic acid (20:4n-6). Canola oil feeding led to a small proportional increase in ileal EPA and DHA and in colonic DHA without significantly affecting contractility. The confirmed depressed PG response in the SHR fed a diet of SF (in the form of coconut oil) was not observed for muscarinic, isoprostane, or autocoid peptide agonists (angiotensin or bradykinin) (Patten *et al.*, 2005b). The fish oil supplemented diet (with high DHA) restored the depressed prostanoid response in the ileum but not the colonic tissue. It is still to be determined whether the differences in tissue reactivity following fish oil feeding is explainable by altered PG receptor properties of ileal and colonic tissue. Functionally, the large intestine is primarily for drying and storage of digestive waste material and a fermentation vat, whereas the contractile properties influenced by n-3 PUFA in the ileum may not be translated to the distal bowel.

Electrically driven contraction of colon is predominantly induced by acetylcholine release (Stanton *et al.*, 2004) and it was demonstrated that there was a significant increase in maximal contraction in the proximal colon by fish oil supplementation compared with a SF diet (Patten *et al.*, 2005b). This observation was supported by a significantly higher acetylcholine-induced contraction of the proximal colon by the fish oil supplemented diet compared with SF diet. This observation was not observed for normotensive SD rats in a previous study (Patten *et al.*, 2002a). Since fish oil has now been demonstrated to stimulate contractility in the large bowel of a rat model with compromised PG function (SHR), this may add weight to the hypothesis that fish oil feeding may be advantageous in conditions where bowel function is compromised such as IBD where it is known contractility is diminished (Menzies *et al.*, 2001). This

is because a main form of IBD, ulcerative colitis, occurs mainly, as its name suggests, in the large bowel.

In an attempt to determine the mechanism of the n-3 PUFA effects on gut contractility, muscarinic binding properties were measured in ileal membrane preparations to determine whether fish oil supplementation modified ileal muscarinic receptor characteristics. Although fish oil feeding markedly increased ileal maximal contraction compared with an SF supplemented diet in response to acetylcholine, there was no change in the muscarinic receptor population measured by the non-selective muscarinic antagonist, [³H]-quinuclidinyl benzylate (Patten *et al.*, 2005b). Any change in muscarinic receptor subtypes was yet to be determined. It may be that the n-3 PUFA modification of contractility is a post receptor mechanism and involves altered calcium handling (Triboulot *et al.*, 2001).

Supplementing SHR with 5% fish oil as HiDHA[®] (w/w) resulted in a significant increase in the proportion of membrane total phospholipid as DHA of ileum (9.7%) and colon (9.9%) with no detectable amounts of EPA found. The addition of 5% (w/w) canola oil to the diet which provided 10.2% (w/w) of the dietary FA as ALA, resulted in only a small conversion to DHA from baseline of 1.5% and 1.8% to 2.6% and 2.9% of the proportion of total fatty acids in membrane total phospholipids of ileum and colon, respectively (Patten *et al.*, 2005b). This relatively small increase in the proportion of the gut compared with that for fish oil supplemented rats. It is to be determined whether diets containing higher levels of EPA or ALA delivered as oils or as pure methyl- or ethyl-esters can be converted into sufficient membrane DHA to

significantly alter contractility. Until such studies can be carried out, it appeared that DHA was likely the active agent underlying the fish oil increase of gut contractility parameters. Interestingly, a more prominent role of DHA rather than EPA has been found with regard to modifying vascular reactivity and lowering blood pressure (Mori *et al.*, 1999a, 2000, 2006). However, the critical level of membrane DHA in gut tissue had yet to be determined.

The level of membrane DHA in gut tissue that can influence contractility is an important issue. Previous experiments were conducted at dietary levels around 1.5% fish oil (w/w) of the total diet for the guinea pig (Patten *et al.*, 2002b) and from 5-10% (w/w) for the rat (Patten *et al.*, 2002a, 2005b) for periods of between 4 and 12 weeks. This resulted in increased total phospholipid n-3 PUFA incorporation into gut tissue which was dependent on the ratio of EPA to DHA in the fish oil dietary supplement. If one assumes that an average adult male eats about 800 g of food per day, 5% fish oil (w/w) in the diet would equate to 40 g of fish oil. This would be well above the typical consumption of sources of n-3 PUFAs be they derived from capsule or from the fish meal itself.

2.7. Dietary fish oil dose effects in WKY rats

The aim of the next study was to evaluate a dosage range for supplemented dietary fish oil rich in DHA fed for 4 weeks on ileal n-3 PUFA levels and effects on nonreceptor- and receptor-induced ileal contractility in normotensive WKY rats. A previous time and dose study had demonstrated that erythrocyte and cardiac membrane n-3 PUFA concentrations derived from dietary fish oil supplementation

were maximal at 4 weeks (Owen *et al.*, 2004) with significant increases in tissue total phospholipid n-3 PUFA evident at 1.25% (w/w) dietary fish oil.

Groups of ten to twelve 13-week old WKY were fed 0, 1, 2.5 and 5% (w/w) fish oil supplemented diets balanced with sunflower seed oil. For the total phospholipid fraction, increasing the dietary fish oil levels led to a significant increase first evident at 1% fish oil, with a stepwise, non-saturating, six-fold increase in n-3 PUFA present as EPA, DPA and DHA, but mainly as DHA replacing the n-6 PUFA linoleic acid (18:2n-6) in ileal phospholipids. There was no difference in KCl-induced depolarization-driven contractility. However, a significant increase in receptor-dependent maximal contractility occurred at 1% (w/w) fish oil for carbachol (a muscarinic mimetic) and at 2.5% (w/w) fish oil for PGE₂, with a concomitant increase in sensitivity to PGE₂ at 2.5 and 5% fish oil supplementation. These results demonstrate that those significant increases in ileal membrane n-3 PUFAs occurred at relatively low doses of dietary fish oil, with differential receptor-dependent increases in contractility being present for muscarinic and prostanoid agonists (Patten *et al.*, 2005b).

The time required for a certain dose of dietary fish oil to significantly increase gut modulator-induced contractility is presently unresolved. All studies to date have involved the feeding of fish oil supplemented diets for four weeks or longer Patten *et al.*, 2005b). Other studies have concluded in cardiac tissue for example, that n-3 PUFA content reached a maximum at around four weeks of feeding (Owen *et al.*, 2004). There are unpublished findings (Patten *et al.* 2005) that indicated that a significant increase in rat ileal contractility had not occurred after two weeks of fish

oil gavage, despite a significant increase of n-3 PUFA in total membrane phospholipids.

2.8. Interactive effects of resistant starch and fish oil in young SD rats

It had been independently established that dietary fibre (Patten et al., 2004b) and fish oil modulate gut contractility of in vitro intact gut tissue from normal and hypertensive small animal models (Patten et al., 2002a,b; 2005a,b). These findings complement experimental and epidemiological data that dietary fibre and resistant starch promotes large bowel function through faecal bulking and greater production of SCFA through bacterial fermentation (Topping and Clifton, 2001; Henningsson et al., 2003). The final study in the series (Patten et al., 2006) investigated the interactive effects of resistant starch (RS) as high amylose maize starch (HAMS) and tuna fish oil on ileal contractility in young SD rats. Four-week old rats were fed diets for 4 weeks containing 100g/kg fat as sunflower oil or tuna fish oil (HiDHA[®]), with 10% fibre (w/w) as α -cellulose or as HAMS (Patten *et al.*, 2006). Previous results showed no difference in total muscarinic receptor population of a crude ileal membrane fraction after fish oil feeding compared to SF (Patten et al., 2005b). In this study, dietary effects on ileal muscarinic receptor subtypes were examined physiologically in the organ bath by pre-contracting tissue with carbachol at a dose approximating the EC_{50} and adding specific muscarinic inhibitors (denoted by subscripts), atropine sulphate (M_{123}) , pirenzepine dihydrochloride (M_1) , methoctramine tetrahydrochloride (M_2) or 4-diphenylacetoxy-N-methyl-piperidine methobromide (M₃) to block contraction.

In the first instance, fish oil feeding led to higher proportions of the ileal n-3 fatty acid levels (mainly as DHA) and greater agonist-induced maximal contractility with a RS

effect noted following carbachol stimulation (Patten *et al.*, 2006). HAMS-containing diets resulted in lower colonic pH and higher SCFA levels except for butyrate following fish oil supplementation. Interestingly, low prostanoid responses were found in young rats compared to older groups (Patten *et al.*, 2004a) and these responses were not evident for muscarinic or isoprostane responses. This blunted response was greatly enhanced by n-3 PUFAs from fish oil and following a fish oil plus RS dietary supplementation. A depressed prostanoid response has recently been reported for SHR (Patten *et al.*, 2004a) which could be increased by dietary fish oil in the ileum but not the colon; even though ileum and colon incorporated similar proportions of n-3 PUFA, mainly as DHA, into the total phospholipid pools (Patten *et al.*, 2006).

Physiological recordings indicated that the order of muscarinic subtype responses in the young rats were different with rank order potency of inhibition being $M_3 > M_1 >$ M_2 compared to that reported in older rats of $M_3 > M_2 > M_1$ (Sales *et al.*, 1997). It has also been reported that there are maturational changes of the muscarinic receptor subtypes and their coupling to G proteins in rat colonic and ovine ileal smooth muscle (Zhang, 1996). Furthermore, it was reported that the sensitivity of the M_1 receptor subtype was modified significantly by fish oil supplementation of the diet (Patten *et al.*, 2006). In respect to this, fish oil feeding of specifically DHA has been reported to augment the muscarinic agonist-induced chloride secretion in human intestinal T84 cells (Del Castillo *et al.*, 2003) and to increase the expression of M_1 muscarinic receptor subtype in the NG108-15 neuroblastoma cell line (Machova *et al.*, 2006) which lead to an elevated [Ca²⁺]_i level in response to increased intracellular cyclic ADP-ribose after the addition of acetylcholine (Higashida *et al.*, 2007). Further, n-3 PUFA has also been found to increase brain blood flow with a concomitant increase in the density and binding characteristics of hippocampal M₁ muscarinic cholinergic receptors (Farkas *et al.*, 2002. It is, therefore, possible that n-3 PUFAs incorporated into gut smooth muscle cell membranes increased ileal contractility by a postmuscarinic receptor mechanism. This may involve the triggering of IP₃ hydrolysis via phospholipase C and increased cGMP levels resulting in increased calcium mobilization and an increase in the ability of gut to contract (Ehlert *et al.*, 1999; Oyachi *et al.*, 2000; Unno *et al.*, 2000).

In general, the n-3 PUFAs from marine sources may be beneficial in the early development of atopic diseases (Duchen and Bjorksten, 2001) such as asthma (Oddy *et al.*, 2004), for diabetes risk (Balck *et al.*, 1993; Nelson and Hickey, 2004), for the inflammatory response in children with arthritis (Alpigiani *et al.*, 1996), and ulcerative colitis (Belluzzi, 2004). This adds to the well documented effects of n-3 PUFAs in CVD and prevention of sudden cardiac death (Psota *et al.*, 2006), and for the proposed protection and prophylaxis of bowel cancer in older populations (Roynette et al., 2004; Chapkin *et al.*, 2007a,b). However, the role for dietary fibre and fish oil in normal and pathophysiological GI conditions warrants further study (Belluzzi, 2004; Toden *et al.*, 2007).

2.9. Summary of major experimental findings relating to this thesis

A schematic representation of the major findings relating to this thesis is shown in **Figure 12**. A comprehensive list of the findings of this thesis is given as follows:

- In the modified organ bath apparatus with a suspended section of intact guinea pig ileum, the luminally-applied opioid antagonists, casoxin 4 and [D-Ala²]casoxin 4, overcame morphine inhibition of electrically-driven contractions.
- For a brown congee supplemented diet, a small but significantly higher voltage was required to initiate guinea pig ileal contraction compared to control fibre dietary groups supplemented with egg custard or baked beans.
- Dietary tuna fish oil supplementation of ~ 1.5% (w/w) for two months increased guinea pig ileal total membrane phospholipid n-3 PUFA content (ALA, EPA and DHA) while lowering the sensitivity to electrically-driven contractions and the isoprostane, 8-*iso*-PGE₂.
- Rats fed diets supplemented with 17% (w/w) dietary fat that included 10% fish oil (w/w) high in EPA for four weeks had higher maximal contractions up to 93% induced by acetylcholine, 8-*iso*-PGE₂, PGE₂, PGF_{2α} and the thromboxane A₂ mimetic, U-46619, compared to comparable Sunola oil (high in 18:1n-9) and saturated fat (SF) supplemented groups, with no changes in sensitivity (EC₅₀).
- The fish oil supplemented dietary group had increased proportions of n-3 PUFAs EPA, DPA and DHA, incorporated into the total membrane phospholipid pool of rat ileum and colon, with the colon registering no changes in reactivity to any of the muscarinic or eicosanoid agonists.
- In the spontaneously hypertensive rat (SHR) and WKY controls, increasing dietary supplementation with SF as lard from 0-27% (w/w) had no effect on ileal contractility, with changes in colonic sensitivity noted for angiotensin II in WKY and carbachol (muscarinic mimetic) in SHR.

- When the three SF dietary groups were combined, there was lower sensitivity and lower maximal contraction in ileum and lower maximal contraction in colon of SHR in response to PGE_2 and $PGF_{2\alpha}$ compared with the WKY control.
- ► In a subsequent study, adult SHR were fed diets supplemented with 5% (w/w) fat as coconut oil, lard, canola oil or fish oil (rich in DHA) and WKY 5% coconut oil for twelve weeks. Contractility studies confirmed a lower gut response to the prostanoids, PGE₂ and PGF_{2α}, in SHR compared to WKY supplemented with coconut oil.
- > In the SHR, fish oil dietary supplementation increased the total membrane phospholipid n-3 PUFA pool as DHA, increased the maximal contraction response to acetylcholine in ileum (up to 89%) compared to all other dietary groups, and increased the contraction of the colon compared to lard (40%), without changes in total muscarinic binding in a crude membrane fraction of the ileum. Fish oil supplementation also restored the depressed contraction in response to PGE₂ and PGF_{2a} in the ileum but not colon of SHR.
- For the proximal colon of SHR, dietary fish oil supplementation increased the maximal electronically-induced contraction (71%) compared to the SF group.
- In adult WKY rats fed 0, 1, 2.5 and 5% fish oil (w/w) supplemented diets for four weeks, there was a significant stepwise, non-saturating, six-fold increase in the proportion of total ileal membrane phospholipid n-3 PUFA mainly as DHA replacing LA and AA, with no difference in the non-receptor, KClinduced depolarization-driven contraction, with a significant increase in maximal contraction at 1% fish oil for carbachol and 2.5% fish oil for PGE₂.



12. Schematic diagram of major experimental findings from this thesis.

- In the final study, young SD rats were fed a dietary supplementation matrix of 10% (w/w) α-cellulose or high-amylose maize starch (HAMS) as resistant starch (RS) or 10% fat (w/w) fat as sunflower oil or tuna fish oil for six weeks to investigate potential interactive effects of fibre and oil. Fish oil supplementation, as expected, led to higher n-3 FA levels (mainly as DHA) in the ileal membrane total phospholipid pool and higher agonist-induced maximal contractility with an RS effect noted for carbachol.
- HAMS-containing diets resulted in lower colonic pH and high SCFAs (but not butyrate) with fish oil.
- > The low prostanoid (PGE₂ and PGF_{2 α}) responses described for young rats were enhanced by dietary fish oil.

- The order of muscarinic receptors subtype rank order potency responses of young rats M₃ > M₁ > M₂ were different compared to older rats M₃ > M₂ > M₁; with fish oil feeding altering the sensitivity of the M₁ receptor subtype.
- Evidence suggests that dietary fish oil as DHA may alter rat and guinea pig receptor-driven gut contractility that probably involves pre- (electricallydriven) and post-receptor (agonist-induced) mechanisms that involve modulation of gut smooth muscle calcium mobilization.

2.10. Future studies

It has been established that the effective dietary concentration of fish oil to achieve a significant increase of *in vitro* rat ileal contractility is between 1-2.5% (w/w) of the total diet when fed for 4 weeks. In fish oil gavage studies of only two weeks duration, it was found that whilst ileal total phospholipid membrane n-3 PUFA content had increased significantly to mirror these levels in the dietary studies described above, a significant increase in muscarinic and prostanoid-induced contractility had not yet been achieved (Patten *et al.*, unpublished observation). Rigorous dose and time course studies, therefore, need to be conducted.

Experiments described herein have also shown that eicosanoids (PGE₂, PGF_{2 α}, 8-*iso*-PGE₂ and U-46619) modulate ileal contractility when guinea pigs and rats are fed diets supplemented with fish oil (Patten *et al.*, 2002a,b; 2005a, 2006). It has also been demonstrated that there is a depressed prostanoid response in gut tissue from the SHR model compared to the WKY control group that is restored by dietary fish oil supplementation (Patten *et al.*, 2004a, 2005b). The status of the prostaglandin receptor expression could be explored using mRNA probes for binding PGE₂ to gut tissue for

the four member EP family of prostanoid receptors (Cosme *et al.*, 2000) from SHR and healthy groups of rats fed diets supplemented with fish oil or saturated fat (as control). More specifically, membrane preparations from ileum and colon could also be prepared from rats fed diets supplemented with fish oil for radioligand binding studies using [³H]-PGE₂ and [³H]-17-phenyl-PGF_{2α} using unlabelled PGE₂ and PGF_{2α} and appropriate prostanoid antagonists to characterize specific binding (Woodward et al., 1995).

From mechanistic experiments where muscarinic receptor systems are modulated, it appears that fish oil may be affecting Ca^{2+} handling from extra- and/or intracellular pools. This may explain why membrane incorporation of n-3 PUFAs, probably with DHA as the active agent, increased contractility of ileum in healthy rats and ileum and colon of SHR where defects in prostanoid mechanisms were apparent. The role of Ca^{2+} could be investigated in the isolated organ bath system by regulating external and internal calcium uptake and release mechanisms by the use of specific inhibitors acting at specific points of Ca^{2+} regulation. The specific role of individual n-3 PUFAs could be tested in isolated intestinal smooth muscle cells from rats fed diets supplemented with fish oil or purified EPA, DHA or ALA to trace ionic movements using radioactive or fluorescent technologies analogous to experiments described for isolated cardiomyocytes (Leifert *et al.*, 1999, 2000b, 2001; Xiao *et al.*, 2005; Jahangiri *et al.*, 2006).

In various small animal models of IBD, interventions with interleukins, fibre or fish oil supplemented diets have improved histological, biochemical and contractile indicators (Vilaseca *et al.*, 1990; Kanauchi *et al.*, 1998; Nieto *et al.*, 2002; Depoortere

et al., 2000; Greenwood-Van Meerveld *et al.*, 2001; Moreau et al., 2003, 2004; Araki et al., 2007). The first report of improvement of contraction by a cytokine, human recombinant IL-11 (rhIL-11), in a genetically-induced model of colitis where contractility was compromised was demonstrated by Greenwood-Van Meerveld *et al.*, 2001, and is shown in **Figure 13**. To date, no laboratory has reported that dietary fish oil intervention improves the disease activity index as well as increases impaired contractility in an animal model of colitis. However, an inflammatory model of colitis

NOTE: This figure is included on page 64 of the print copy of the thesis held in the University of Adelaide Library.

Figure 13. Genetically-induced gut inflammatory model. Concentration dependent contractions induced by carbachol (CCh) in longitudinal muscles isolated from the jejunum (A) and colon (B) of F344 rats that received placebo (\Box) or HLA-B27 transgenic rats with chronic intestinal inflammation that received oral doses of either placebo (•) or oral enteric coated recombinant IL-11 (diagonally filled square). From Greenwood-Van Meerveld *et al.*, 2001.



Figure 14. Chemically-induced (DSS) model of gut inflammation. Sprague-Dawley rats were dosed with 2% DSS in the drinking water for 5 (black) or 7 days (red), or control (green). The effects on contraction using electrical stimulation (60 V, for 5 msec at 0.02 Hz) or 30 mM KCI-induced contraction of are shown for colon (A) or ileum (C) or concentration-dependent carbachol-induced contractions of colon (B) or ileum (D). Results are means \pm SEM for n = 3-5 rats.

using DSS in the rat has been established at CSIRO Human Nutrition where colitis severity scores have been correlated with contractility dysfunction and muscarinic binding properties (Patten *et al.*, 2005b, Patten *et al.*, unpublished observations, see **Figure 14**, **Figure 15**). Properly controlled dietary studies could be conducted in which fish oil dietary supplementation (shown at CSIRO Human Nutrition to increase gut contractility of ileum and colon, Patten *et al.*, 2002a, 2005b, 2006; see **Figure 16** for example) is included before, during or after the experimental induction of colitis in an established rat model to test fish oil efficacy on improving disease activity index

and contractility outcomes. Since mucosal tissue concentrations of PGE₂ are heightened 3-fold during mucosal inflammation, the status of the prostaglandin receptor expression could again be explored using receptor specific mRNA probes and radioligand binding studies using [³H]-PGE₂ and [³H]-17-phenyl-PGF_{2α} (Woodward *et al.*, 1995; Cosme *et al*, 2000; Mutoh *et al.*, 2006). From recent experiments described in the literature and results reported herein, cofactors such as



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Figure 15. Muscarinic receptor binding to DSS-treated rat colon membranes. Saturation binding isotherms for the tritiated muscarinic antagonist quinuclidynil benzilate ([³H]-QNB) from the pooled data from colon tissues (3000-46000 x *g* sub-fractionation) from n =3-5 rats treated with water (•), or treated with 2% DSS in the drinking water for 5 days (•) or 7 days (•). Results are plotted at each concentration as mean \pm SEM.

antioxidants, fibre (as resistant starch) as well as probiotics/probiotics (Geier *et al.*, 2007; Hedin *et al.*, 2007; Peran *et al.*, 2007), could be factored into the experimental matrix to maximize the potential effects of fish oil. While the beneficial effects of the n-3 PUFAs on CVD and cancer have been reported to varying degrees, and are still

controversial (Schmidt *et al.*, 2006; Roynette *et al.*, 2007), much remains to be done in others areas in which the effects of n-3 PUFAs may be more subtle, and require other dietary cofactors to provide optimal beneficial outcomes. Thus the GI tract represents just such an area.

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Figure 16. Effects of dietary fatty acid supplementation on agonist induced gut contractility of Sprague-Dawley rats. The effects of 17% total fat diet (w/w) as Sunola oil substituted with 10% fat as Sunola oil control (SO, \blacktriangle), saturated fat (SF, \blacksquare) or fish oil (FO, **o**) is shown for concentration-dependent agonist-induced contraction by acetylcholine (Ach) in colon (A) or ileum (B) or for the isoprostane, 8-*iso*-PGE₂, in colon (C) or ileum (D). Results are means \pm SEM for n = 5-8 rats per dietary group. Significance is indicated by * *P* < 0.05. From Patten *et al.*, 2002b.

"An expert is a man who has made all the mistakes, which can be made, in a very narrow field." *Niels Bohr*.

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