DIETARY POLYUNSATURATED FATTY ACIDS AND VASCULAR AND CARDIAC RESPONSES TO ALPHA1-ADRENOCEPTOR STIMULATION

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Declaration

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I hereby declare that the experimental work included in this thesis was undertaken by myself during the tenure of a British Heart Foundation Research Fellowship and Ph.D. studentship in the Cardiovascular Research Unit, Department of Medicine, University of Edinburgh. I was the principal contributor to all sections except where indicated in the text.

Signature *30th March 1993*

Acknowledgements

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This work depended entirely upon the financial support of the British Heart Foundation, to whom I express my thanks.

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I take great pleasure in dedicating this thesis to my parents who, though at times bemused by my research activities, have been unhesitating with their support over the years.

Abbreviations

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BP ..... *blood pressure*

LVP ..... *left ventricular pressure*

LVdP.dt^{-1} ..... *first derivative of LVP*

PC ..... *phosphatidylcholine*

PE ..... *phosphatidylethanolamine*

PI ..... *phosphatidylinositol*

PS ..... *phosphatidylserine*

PUFA ..... *polyunsaturated fatty acids*

P/S ratio ..... *PUFA/saturated fatty acid ratio*

PGI ..... *prostacyclin*

TXA ..... *thromboxane*

18:2, n-6 ..... *linoleic acid*

20:4, n-6 ..... *arachidonic acid*

20:5, n-3 ..... *eicosapentaenoic acid*

22:6, n-3 ..... *docosahexaenoic acid*

The abbreviated nomenclature for individual fatty acids is explained in section 2.7.4.

Abstract

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There is epidemiological evidence that the benefits of dietary polyunsaturated fatty acids (PUFA) derived from fish oil (n-3 PUFA) and plant seed oil (n-6 PUFA) in cardiovascular disease include a reduction in blood pressure (BP). In man, intervention studies have shown that dietary PUFA can affect vascular resistance, an important determinant of BP. Animal experiments have suggested that n-3 PUFA may affect vascular responses to α_1-adrenoceptor stimulation but differences between species, in experimental diets and duration of feeding make interpretation of the results difficult. The aim of this thesis was to use semisynthetic isocalorific experimental diets, representative of what is or could be consumed by man, to investigate the effects of dietary PUFA on cardiovascular responses to α_1-adrenoceptor stimulation.

Generally, diets derived 40% of total calories from fat. The control diet had a PUFA/saturated fat (P/S) ratio of 0.3. Dietary n-3 PUFA were given as a small daily supplement (0.4% dietary calories) to the control diet and the n-6 PUFA diet had a P/S ratio of 2.0, achieved by substituting 18:2, n-6 for saturated fat. In one series of experiments, diets deriving 10% of total calories from fat, relatively rich in saturated fat, n-3 PUFA or n-6 PUFA, were used. Throughout, diets were fed for eight weeks. There were three experimental preparations: the buffer perfused rat hind-quarters, the isolated rat femoral resistance artery and the isolated rat heart retrogradely perfused with buffer through the aorta. Responses to α_1-adrenoceptor stimulation were elicited by noradrenaline in the presence of β- and α_2-adrenoceptor antagonists, where appropriate. The effect of the diets on total and fractionated phospholipid (PL) FA composition was monitored in cardiac tissue.

In the perfused hind-quarters and isolated resistance artery, n-3 PUFA, but not n-6 PUFA, significantly attenuated responses to α_1-adrenoceptor stimulation. A supplementary study in the resistance artery did not reveal any effect of dietary PUFA on relaxation to acetylcholine. In the isolated heart, a 10% fat n-3 PUFA diet, but not

an n-6 PUFA diet, significantly attenuated peak left ventricular pressure (LVP) responses to α_1-adrenoceptor stimulation. The peak LVP effect of n-3 PUFA was maintained after chemical denervation. At 40% fat, the n-6 PUFA significantly attenuated peak LVP responses whereas the n-3 PUFA supplement did not. $LVdP.dt^{-1}$, heart rate and coronary flow were not affected by diet. Parenteral flurbiprofen removed the effect of the 40% fat n-6 PUFA diet on peak LVP. Baseline cardiac prostacyclin release decreased 75% with flurbiprofen and was not influenced by diet.

Analysis of PL FA composition revealed numerous significant changes in relation to diet. In phosphatidylcholine (PC) and phosphatidylethanolamine (PE), dietary n-3 PUFA reduced the proportion of 18:2, n-6 and 20:4, n-6 and increased that of 20:5, n-3 and 22:6, n-3. The n-6:n-3 ratio fell and the double bond index rose. These effects were more profound with the 10% than the 40% fat diets. Phosphatidylinositol (PI) and phosphatidylserine (PS) were more resistant to dietary influence. Dietary n-6 PUFA led to modest increases in 18:2, n-6 and the n-6:n-3 ratio but the double bond index was unaltered or decreased. The proportion of 20:4, n-6 was unaffected by dietary n-6 PUFA at 40% fat and fell paradoxically at 10% fat. No correlation was identified between PL FA composition and the responses of peak LVP.

The influence of the 40% fat diets on aortic *versus* cardiac PL FA composition was investigated. Aortic PC incorporated less 20:4, n-6, and PE more, than the heart. All aortic fractions incorporated more 20:5, n-3 and less 22:6, n-3 than the heart. Levels of 18:2, n-6 were broadly similar. Higher n-6:n-3 ratios and lower double bond indices were further evidence of lesser incorporation of longer chain PUFA in the aorta.

In conclusion, dietary PUFA reduced vascular and cardiac responses to α_1-adrenoceptor stimulation. Dietary PUFA altered cardiac PL FA composition but no direct relationship could be demonstrated between FA composition and cardiac responses. Dietary PUFA did not affect baseline cardiac prostacyclin release but the attenuatory effect of dietary n-6 PUFA on cardiac peak LVP responses was removed

by flurbiprofen. The influence of n-3 PUFA on vascular responses may help to explain BP lowering effects of fish oil in man.

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Chapter 1

Introduction

Cardiovascular disease continues to be the principal cause of death in the developed world. In 1987, diseases of the heart and circulation accounted for one half of all mortality [Lopez, 1990], a proportion which had altered little during the previous decade [Balajaran, 1991, Kannel *et al.*, 1984]. Coronary heart disease alone is responsible for a quarter of deaths. Hypertension, raised BP, is not associated with a high incidence of mortality, but is a major risk factor for other cardiovascular diseases. Moreover, hypertension is eight times more prevalent than coronary heart disease [Kannel *et al.*, 1984] and is thus a substantial source of morbidity. Recognition of the importance of detecting and treating hypertension is accompanied by increasing interest in non-pharmacological means of management, including the use of PUFA derived from plant seed oils (n-6 PUFA) and fish oils (n-3 PUFA). This Introduction will begin by considering the epidemiological evidence that dietary PUFA are associated with lower BP.

1.1 Dietary PUFA and blood pressure

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1.1.1 Population studies

Evidence suggesting that dietary PUFA may be associated with lower BP can be found in a number of epidemiological studies. These studies were commonly performed to assess risk factors within populations who differed in their prevalence and incidence of coronary heart disease. Thus, Scottish men were found to have higher systolic and

diastolic BP, and lower adipose tissue 18:2, n-6, than Swedish men [Logan *et al.*, 1976]. Likewise, Italian men were noted to have lower systolic and diastolic BP than their Scandinavian counterparts [Riemersma *et al.*, 1986, Olsson *et al.*, 1988]. The first of these studies revealed that the Italians had a higher proportion of adipose tissue 18:2, n-6 and the proposal that this finding was consistent with a higher dietary P/S ratio was confirmed in the second study. In an investigation of serum phospholipid fatty acid composition in Finnish men sustaining a myocardial infarction, 18:2, n-6 was negatively correlated with systolic and diastolic BP in the control group [Miettinen *et al.*, 1982]. Weak, but significant, negative correlations were found between adipose tissue 18:2, n-6 and platelet 20:5, n-3 in a control group of middle-aged Edinburgh men [Wood *et al.*, 1987]. Reviewing several epidemiological studies, mostly performed in Greenland Eskimos, Bønaa [1989] concluded that BP tended to be lower in relation to increased consumption of n-3 PUFA.

In response to these observations, a number of dietary intervention studies, discussed in the following section, have been carried out to investigate further the potential BP lowering effects of n-6 PUFA and n-3 PUFA in man.

1.1.2 Dietary intervention studies and BP in man.

Studies using dietary intervention to investigate the effect of PUFA on BP do so in two ways. The overall proportion of PUFA in the diet can be increased at the expense of saturated fat or the diet can be supplemented with the required PUFA. The majority of such studies concern n-3 PUFA and, curiously, have been performed relatively infrequently in hypertensive subjects.

Systolic BP was reduced by a mackerel diet in individuals with mild essential hypertension [Singer *et al.*, 1985]. Likewise, dietary supplements providing approximately 5 g [Norris *et al.*, 1986] or 15 g [Knapp and Fitzgerald, 1989] of n-3 PUFA daily led to small but significant falls in BP in mild hypertension. The combination of a decrease in dietary fat intake (to 23% total calories) and an increase in dietary n-6 PUFA (to a P/S ratio of 1.0) was found to reduce BP, more so for hypertensives than normotensives, in Finnish men [Puska *et al.*, 1983].

In normotensive men, n-3 PUFA given as fish oil [Mortensen *et al.*, 1983] and fish paste [van Houwelingen *et al.*, 1987] reduced systolic BP, although in the latter study, BP also fell with meat paste containing a high proportion of 18:1, n-9. Studies examining the effects of dietary n-6 PUFA on BP in healthy volunteers have not been consistent. A low fat diet (24% total calories) with an increased proportion of n-6 PUFA (P/S ratio 1.2) lowered systolic and diastolic BP in normotensive Finns [Iacono *et al.*, 1983] and dietary supplementation with safflower seed oil (a source of 18:2, n-6) reduced systolic BP in healthy males [Heagerty *et al.*, 1986]. However, diets with a P/S ratio of 1.0 had no effect on BP in either 54 Australian or 58 Dutch volunteers [Margetts *et al.*, 1985, Mensink *et al.*, 1990]. There are no appreciable differences in systolic or diastolic BP at the outset in these four n-6 PUFA studies.

Animal intervention studies, where diets can be tightly controlled, and BP measured frequently under reproducible conditions, might be expected to confirm the effects of n-3 PUFA and clarify those of n-6 PUFA. This area is discussed below.

1.1.3 Dietary intervention studies and BP in animals

In contrast to the general trend in man, the effects of dietary n-3 PUFA on BP in various animal models have not been consistent. Dexamethasone-induced hypertension was attenuated in rats fed a diet supplemented with cod liver oil (another source of n-3 PUFA) as opposed to saturated fat [Codde and Beilin, 1985], but the same research group found that spontaneously hypertensive rats showed a non-sustained increase in BP with fish oil treatment [Croft *et al.*, 1988]. Rats rendered hypertensive with oral 1.5% saline increased their BP less in relation to dietary cod liver oil than sunflower seed oil (n-6 PUFA), and the rise in BP was less for both series of PUFA compared to a saturated fat diet [Ziemlanski *et al.*, 1985].

In normotensive animals, reports of the effects of n-3 PUFA are equally conflicting. Cod liver oil was associated with raised BP in rats after six weeks [Scherhag *et al.*, 1982] but, interestingly, 20:5, n-3 delivered by an osmotic pump blunted the increase in BP in rats subjected to isolation stress [Mills and Ward, 1986].

The few data regarding the effects of n-6 PUFA in animal models tend to agree, in contrast to the human studies. In one-clip one-kidney (Goldblatt) hypertensive rats, sunflower seed oil curtailed the rise in BP [Mahoney *et al.*, 1983]. The similar results of Ziemlanski *et al.* [1985] with regard to sunflower seed oil and saline-induced hypertension in the rat are mentioned above. Also, normotensive rats sustained lower BP after feeding with sunflower seed oil compared to saturated fat [Charnock *et al.*, 1985].

An important observation when considering the effect of experimental diets on BP in animal studies is that dietary 18:2, n-6 reverses the relative hypertension that occurs with essential fatty acid deficiency [Cox *et al.*, 1982]. The reason for the inconsistent nature of the results in n-3 PUFA dietary experiments may be that some of the diets used in these studies were deficient in n-6 essential fatty acids.

A number of mechanisms have been proposed to underlie the effects of dietary PUFA on BP in human and animal work and these are discussed below.

1.1.4 Putative mechanisms in the effect of dietary PUFA on BP: studies in man

Virtually all the mechanisms thought to account for the effects of dietary PUFA in human studies are in relation to the effects of n-3 PUFA. Indeed, most concern factors likely to influence peripheral vascular resistance, an important determinant of BP. (BP is the product of cardiac output and peripheral vascular resistance.)

The tendency for BP to fall with dietary n-3 PUFA is accompanied by either unchanged [Mortensen *et al.*, 1983] or increased [Singer *et al.*, 1985, Jørgensen *et al.*, 1986] renin levels. If hypotensive effects of n-3 PUFA were associated with a decrease in renal perfusion, renin levels would be expected to rise, but an alternative explanation involves decreased sensitivity to the powerful vasoconstrictor angiotensin II, as was found in volunteers given purified 20:5, n-3 [Yoshimura *et al.*, 1987].

There are conflicting reports concerning the effects of dietary n-3 PUFA on eicosanoids. Some authors describe decreases in PGI_2 and TXA_2 synthesis from 20:4, n-6 with increases in PGI_3 and TXA_3 synthesis from 20:5, n-3 [Lorenz *et al.*, 1983, Fischer and Weber, 1983, Fischer and Weber, 1984, Knapp and Fitzgerald, 1989].

Others describe a simple decrease in platelet cyclooxygenase/lipoxygenase products [Driss *et al.*, 1984]. Even a paradoxical increase in PGI_2 synthesis following dietary n-3 PUFA has been reported [de Caterina *et al.*, 1990]. Generally, as TXA_3 is a less potent vasoconstrictor than TXA_2, but PGI_3 and PGI_2 equipotent vasodilators, dietary n-3 PUFA shift the balance in favour of vasodilator prostanoids.

Perhaps the best known effects of n-3 PUFA are on blood rheology. Decreased viscosity, increased erythrocyte deformability and decreased erythrocyte and platelet aggregation have all been reported [Terano *et al.*, 1983, Cartwright *et al.*, 1985, Driss *et al.*, 1984, Ernst, 1989]. A reduction in fibrinogen, an important determinant of plasma viscosity, has also been found with n-3 PUFA [Høstmark *et al.*, 1988]. These changes would contribute to a fall in BP.

There may be an effect of dietary n-3 PUFA on the mechanical characteristics of conduit vessels. A decrease in aortic pulse wave velocity, measured indirectly by means of Doppler ultrasound, in a fish-eating population was proposed to reflect an increase in vascular compliance [Hamazaki *et al.*, 1988]. Similar results were obtained in non-insulin-dependent diabetics and healthy controls fed in excess of 100 g fish per week [Wahlqvist *et al.*, 1989].

Potential effects of dietary n-3 PUFA on vascular reactivity attract increasing interest. Attenuated pressor responses to noradrenaline [Lorenz *et al.*, 1983], to angiotensin II [Yoshimura *et al.*, 1987] and even mental stress [Singer *et al.*, 1985] have been reported, though clearly the last study may reflect a central effect of dietary n-3 PUFA on sympathetic activation. Further, the systolic BP response to treadmill exercise testing was reduced in angina sufferers treated with fish oil [Mehta *et al.*, 1988] and the onset of digital vasoconstriction in response to cold provocation was delayed in patients with Raynaud's disease given fish oil. These data are consistent with the concept that dietary n-3 PUFA can attenuate the increase in peripheral vascular resistance brought about by catecholamines or other vasoactive agents.

The mechanisms responsible for hypotensive effects of dietary n-6 PUFA have been little investigated. An increase in leukocyte total Na^+ efflux was found in

association with a fall in systolic BP in mild hypertensives given safflower oil [Heagerty *et al.*, 1986], suggesting that abnormal membrane Na^+ transport in hypertensives may respond to n-6 PUFA. Dietary n-6 PUFA may influence vascular reactivity or sympathetic activation in a similar manner to n-3 PUFA. In healthy volunteers, a diet rich in 20:4, n-6 attenuated the fall in forearm blood flow induced by cold stimulation (ice applied superficial to the carotid bifurcation) [Butcher *et al.*, 1990].

In the following section, mechanisms suggested by animal studies are discussed.

1.1.5 Putative mechanisms in the effect of dietary PUFA on BP: animal studies

Data are sparse regarding the effects of dietary n-6 PUFA and, as in human studies, many of the mechanisms suggested for dietary n-3 PUFA are linked to changes in vascular resistance. Much of the evidence comes from *in vitro* work.

Decreases in circulating PGI_2 and TXA_2 [Codde and Beilin, 1985] and in their synthesis by aortic tissue [Scherhag *et al.*, 1982, Croft *et al.*, 1988] have been found in rats fed n-3 PUFA but in these studies, the effects of n-3 PUFA on BP were inconsistent. The finding of Mills and Ward [1986] that BP responses to isolation stress were attenuated in rats fed fish oil suggests a mechanism involving a decrease in sympathetic activation or in vascular responses to catecholamines or other vasoactive agents, as proposed in some of the human studies.

In animal work also, there is great interest in the potential effects of dietary n-3 PUFA on vascular reactivity. Aortic rings isolated from rats fed fish oil have been shown by more than one research group to have diminished contractile responses to noradrenaline [Lockette *et al.*, 1982, Yin *et al.*, 1991, Malis *et al.*, 1991]. Also, in rabbits, highly purified 20:5, n-3 was associated with reduced pressor responses to angiotensin II and noradrenaline, though the effect on noradrenaline responses did not achieve significance. In marked contrast to these findings, oral dosing with fish oil for one week led to enhanced BP responses to α_1-adrenoceptor stimulation [Kenny *et al.*, 1990]. However, the control animals were not orally dosed and the effect of this

procedure was not examined. Generally, the data indicate a negative effect of dietary n-3 PUFA on vasoconstriction.

The influence of dietary n-3 PUFA on vascular relaxation became clear in animal studies. Coronary artery rings isolated from swine fed cod liver oil demonstrated enhanced relaxation in response to bradykinin, serotonin and adenosine [Shimokawa *et al.*, 1987]. The effect was dose- (of oil) dependent, endothelium-dependent and removed by methylene blue, allowing the authors to conclude that the mechanism involved endothelium-derived relaxing factor. There are similar data from rat studies. Dietary fish oil enhanced the relaxation of aortic rings from spontaneously hypertensive rats [Yin *et al.*, 1988, Yin *et al.*, 1991], an effect thought to have both endothelium-dependent and endothelium-independent components [Malis *et al.*, 1991]. Thus, there are other means by which dietary n-3 PUFA shift the balance in favour of vasodilatation.

As mentioned, there are virtually no animal data concerning mechanisms in n-6 PUFA effects on BP. Reports of increased noradrenaline overflow following field stimulation in the tails of rats fed sunflower seed oil [Panek *et al.*, 1985, Semafuko *et al.*, 1989] are clearly inconsistent with the tendency for n-6 PUFA to reduce BP in other studies.

1.2 Physiological rôles of fatty acids and phospholipids

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As the first part of this Introduction concerned the effects of dietary PUFA on BP, the physiological rôles of fatty acids will be reviewed.

1.2.1 Fatty acids as a source of energy

Our most important source of energy is fat, stored in adipose tissue as triacylglycerols. Fatty acids are highly reduced compared to carbohydrates and yield a correspondingly greater amount of energy on oxidation; 9 $kcal.g^{-1}$ as opposed to 4 $kcal.g^{-1}$ for carbohydrate and protein. Another important difference between fat and carbohydrate as energy sources is the hydrophilic nature of glucose polymers. Glycogen binds

approximately 2 g $H_2O.g^{-1}$ whereas triacylglycerols are anhydrous. Accordingly, intracellular fat contains six times the potential metabolic energy, weight for weight, of intracellular glycogen. Thus a typical 70 kg man has fuel reserves approaching 10^5 kcal in body fat in contrast to some 600 kcal in total glycogen. In response to adrenaline during stress and glucagon when fasting, for example, triacylglycerol is hydrolysed by hormone-sensitive lipase (triacylglycerol lipase) to yield diacylglycerol and one unesterified fatty acid. Diacylglycerol and monoacylglycerol are also hydrolysed by specific lipases. Of the energy subsequently derived from complete oxidation, 95% is from the acyl side chains, 5% from the glycerol backbone.

To undergo degradation, fatty acids in the cytosol are conjugated with coenzyme A (CoA) and transferred onto the carrier, carnitine, to be transported into the mitochondrion where β-oxidation takes place. The process of β-oxidation entails the cyclic release of two-carbon fragments, in the form of acetyl-CoA, commencing at the carboxyl terminal of the fatty acid. One β-oxidation cycle yields 5 molecules of adenosine-*tris*-phosphate (ATP) from reduced electron carriers. The subsequent oxidation of acetyl-CoA in the citric acid cycle yields a further 12 ATP.

The oxidation of unsaturated fatty acids necessitates the conversion of *cis*-Δ^3-enoyl-CoA fragments to *trans*-Δ^2-enoyl-CoA by enoyl-CoA isomerase. After hydration by 2,4-dienoyl reductase, β-oxidation can proceed. These additional steps incur a slight reduction in the overall energy yield.

For more comprehensive details of fatty acid metabolism, the reader may refer to the excellent text of Mathews and van Holde [1990].

1.2.2 Fatty acid synthesis and the essential fatty acids

Generally speaking, endogenous fatty acid synthesis is the reverse of degradation, but the biochemical pathways are quite different. In the cytosol, synthesis commences with acetyl-CoA and malonyl-CoA, both of which are then conjugated to an acyl carrier protein (ACP) to provide acetyl-ACP and malonyl-ACP. Malonyl-ACP serves as an acetyl donor. The condensation of acetyl-ACP and malonyl-ACP produces 3-ketoacyl-ACP which is reduced and dehydrated to butyryl-ACP. Thereafter,

successive molecules of malonyl-ACP are used to lengthen the acyl chain, arriving primarily at 16:0.

To obtain a variety of chain lengths and degrees of unsaturation, elongation and desaturation must take place. In elongation, acyl-CoA receives an acetyl group from malonyl-CoA (not malonyl-ACP) to form 3-ketoacyl-CoA, which is subsequently reduced and dehydrated to a saturated acyl-CoA, two carbons longer than the original. Desaturation requires microsomal enzyme systems; the fatty acyl-CoA desaturases. Mammalian cells contain Δ^4, Δ^5, Δ^6 and Δ^9 desaturases but are unable to introduce double bonds beyond carbon 9 in the fatty acid chain. Consequently, mammals cannot synthesise either 18:2 *cis*-Δ^9,Δ^{12} (18:2, n-6 - linoleic acid) or 18:3 *cis*-Δ^9,$\Delta^{12}\Delta^{15}$ (18:3, n-3 - linolenic acid). These are termed *essential* fatty acids because they must be provided by the diet. Following ingestion, these essential fatty acids themselves become substrates. One pathway of particular importance merits description.

By means of Δ^6 desaturation, elongation and Δ^5 desaturation (*right*), dietary 18:2, n-6 is used to derive 20:4, n-6. This fatty acid species is then preferentially incorporated to the *sn*-2 position in cell membrane phospholipids, PC and PE for example, from where it can be hydrolysed by phospholipase A_2 to act as the precursor for series 2 prostanoid synthesis.

18:2 *cis*-Δ^9,Δ^{12}

⇩ *desaturation*

18:3 *cis*-Δ^6,Δ^9,Δ^{12}

⇩ *elongation*

20:3 *cis*-Δ^8,Δ^{11},Δ^{14}

⇩ *desaturation*

20:4 *cis*-Δ^5,Δ^8,Δ^{11},Δ^{14}

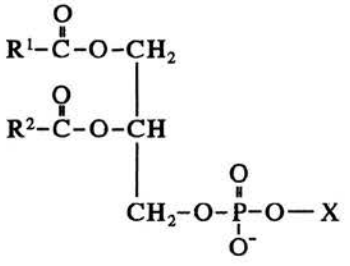
At one time it was believed that the essential fatty acids were solely required to generate substrate for prostanoid synthesis. However, incorporation of unsaturated fatty acids into membrane phospholipids also causes greater separation of *sn*-1 and *sn*-2 chains in the cell membrane lipid bilayer, rendering it more fluid. Consistent with a direct membrane effect, essential fatty acid deficiency in the rat has been shown to decrease myocardial adenylate cyclase activity [Alam *et al.*, 1987] and increase

erythrocyte Na^+/K^+ ATPase and Ca^{2+}/Mg^{2+} ATPase activity [Holmes *et al.*, 1983]. Further, as reviewed by Brenner [1984], there is evidence of negative feedback between the degree of membrane phospholipid unsaturation and Δ^6 and Δ^9 desaturase activity, suggesting a homeostatic mechanism. The rôle of fatty acids and phospholipids in the cell membrane is discussed further in the following section.

1.2.3 Fatty acids and phospholipids in cell membranes

Cell membranes consist of a phospholipid bilayer, approximately 7 nm deep, in which membrane proteins and cholesterol are embedded. The bilayer exists because phospholipids have a polar, hydrophilic head group and apolar, hydrophobic fatty acyl side-chains. A glycerol backbone carries the acyl side-chains at the *sn*-1 and *sn*-2 positions and the polar head group at the *sn*-3 position. The specific nature of the head group distinguishes individual phospholipids (Table 1.1).

TABLE 1.1 *Basic structure, head-groups and nomenclature of membrane phospholipids.*

Basic structure	Head-group (—X)	Phospholipid
	H	phosphatidic acid
	choline	phosphatidylcholine (PC)
	ethanolamine	phosphatidylethanolamine (PE)
	inositol	phosphatidylinositol (PI)
	serine	phosphatidylserine (PS)

R^1 and R^2 denote acyl side chains

Cell membranes are asymmetrical in their phospholipid distribution. In rat liver tissue, for example, PC tends to occupy the outer leaflet of the bilayer whereas PE, PI and PS are more likely to lie in the inner leaflet. This is clearly not a state of equilibrium, as these different components are unequally distributed, and emphasises the dynamic nature of the cell membrane. Current concepts in membrane structure and biochemistry are reviewed in detail elsewhere [Katz, 1992, Hazen and Gross, 1992].

The acyl side chains vary. Commonly, 18:0 or 18:1, n-9 is found at the *sn*-1 position, and 18:2, n-6, or a longer more unsaturated fatty acid species such as 20:4, n-6, at the *sn*-2 position. The orientation of the *sn*-2 chain is such that the first $-CH_2$ group lies parallel to the membrane while the distal chain is perpendicular. It is possible that this arrangement facilitates enzymatic cleavage. The acyl chains at the *sn*-1 and *sn*-2 positions may be cleaved by phospholipase A_1 and A_2 respectively. From the *sn*-2 position, substrate for prostanoid synthesis may be released, as mentioned above, but this is also the means of exchanging one fatty acid species for another.

The influence of dietary PUFA, and of certain other factors such as catecholamines, on phospholipid fatty acid composition has attracted considerable interest because of the links between diet and coronary heart disease, and between cell membrane fatty acid composition and physical properties. Section 1.2.4 discusses this topic.

1.2.4 Dietary PUFA, phospholipid fatty acid composition, and catecholamines

Many of the studies in this area have been performed in the rat and there are several observations germane to this thesis.

Data based on the analysis of tissue *total* phospholipid fatty acid composition may be misleading, as they can obscure important changes, or lack of change, in *fractionated* phospholipid fatty acid composition [Egwin and Kummerow, 1972, Chapkin and Carmichael, 1990]. This is particularly true for the less abundant phospholipid fractions, PI and PS. Diet does not appear to affect the distribution of individual phospholipid fractions within total phospholipid [Swanson and Kinsella, 1986, Benediktsdottir and Gudbjarnason, 1988*a*] or the phospholipid P/S ratio, which is relatively fixed [McMurchie *et al.*, 1983, Sargent, 1990]. Diet instead alters the phospholipid PUFA balance and there are interactions between PUFA of the n-3 and n-6 series. At the level of Δ^6 desaturase, the rate limiting step in fatty acid elongation/desaturation, 18:3, n-3 competes advantageously with 18:2, n-6 [Egwin and Kummerow, 1972, Roshanai and Sanders, 1985]. Also, n-3 series PUFA in general

compete with 20:4, n-6 for esterification at the *sn*-2 position [Iritani and Fujikawa, 1982, Montfoort *et al.*, 1986].

Although 22:6, n-3 is the predominant n-3 PUFA in cardiac and skeletal muscle [Sprecher, 1989, Stubbs and Kisielewski, 1990], it is suggested that these tissues do not synthesis 22:6, n-3 but obtain it from an extracellular source, such as the liver. In cultured myocyte studies, 22:6, n-3 is avidly incorporated but levels are not increased by 20:5, n-3, which can be elongated to 22:5, n-3 but not subsequently (Δ^4) desaturated [Meij *et al.*, 1990, Mohammed *et al.*, 1990].

Significant changes in phospholipid fatty acid composition can occur rapidly with diet. In rats fed fish oil, serum total phospholipid 20:4, n-6 was decreased 23% after 48 hours [Croft *et al.*, 1985]. The fatty acid composition of rat cardiac mitochondria changed in 11 days, and was reversible in the same period, in a soya bean oil/rapeseed oil crossover study [Innis and Clandinin, 1981]. In another study, rat subcellular membranes did not alter further in fatty acid composition beyond three weeks of feeding [Tahin *et al.*, 1981].

Interesting effects of catecholamines on phospholipid fatty acid composition have been described. Chronic treatment of rats with increasing doses of noradrenaline (1-5 $mg.kg^{-1}$ subcutaneously for 2 weeks) brought about reversible decreases in 18:2, n-6 and 20:4, n-6, and increases in 22:6, n-3, in cardiac PC and PE [Emilsson and Gudbjarnason, 1983, Montfoort *et al.*, 1986]. Similar findings with adrenaline occurred regardless of diet (saturated fat, n-6 PUFA or n-3 PUFA) [Benediktsdottir and Gudbjarnason, 1988*b*]. In cultured cardiomyocytes, the fatty acid composition of PI was not affected by exposure of the cells to phenylephrine, but effects on PC and PE were confirmed [Meij *et al.*, 1990].

1.2.5 Specialised rôles of fatty acids: the eicosanoids

The term "eicosanoid" encompasses the prostanoids and leukotrienes. These autacoid families are synthesised from the 20-carbon fatty acids 20:3, n-6, 20:4, n-6 and 20:5, n-3, giving rise respectively to the 1, 2 and 3 series of prostanoids. All three potential substrates must be derived from the essential fatty acids 18:2, n-6 and 18:3, n-3 or

consumed in the diet. Biosynthesis of the prostanoids and leukotrienes is dependent upon the hydrolysis by phospholipase A_2 of fatty acid substrate from the *sn*-2 position of membrane phospholipids, referred to in section 1.2.2. In man, 20:4, n-6 predominates but 20:5, n-3 may be important in relation to n-3 PUFA consumption.

The prostanoids, including prostacyclin (PGI) and thromboxane (TXA), contain ring structures and are derived following the actions of cyclooxygenase on the 20-carbon substrate. Taking the example of 20:4, n-6, the unesterified fatty acid precursor is oxygenated and cyclized to form the unstable cyclic endoperoxide products PGG_2 and PGH_2 ($t_{½}$ 5 minutes at 37°C, pH 7.5). Isomerisation of PGH_2 produces PGD_2, PGE_2 and $PGF_{2\alpha}$. PGH_2 is also metabolised to two biologically very active compounds, PGI_2 and TXA_2. PGI_2, a powerful vasodilator, is characteristically formed in vascular tissue by prostacyclin synthetase, has a $t_{½}$ of 3 minutes (at 37°C, pH 7.5) and is hydrolysed to a stable product, 6-*oxo*-$PGF_{1\alpha}$. TXA_2, a vasoconstrictor and aggregating agent, is typically formed in platelets by thromboxane synthetase, has a very short $t_{½}$ of 30 seconds (at 37°C, pH 7.5) and degrades to the stable TXB_2. The metabolism and functions of prostanoids are reviewed in detail by Moncada and Vane [1979].

Two hydroxylated straight-chain fatty acid products, 12-hydroperoxy-eicosatetraenoic acid (HPETE) and 12-hydroxyeicosatetraenoic acid (HETE), result from the actions of 12-lipoxygenase on 20:4, n-6. The epoxide derivative of HPETE is leukotriene A_4 (LTA_4), from which LTB_4 and a glutathionyl derivative LTC_4 are derived. Removal of γ-glutamic acid from LTC_4 produces LTD_4 and it is now accepted that a combination of these two leukotrienes is the mediator originally termed "slow reacting substance of anaphylaxis". The leukotrienes, which are not of direct relevance to this thesis, are discussed in greater detail in the review of Piper [1983].

PGI_2 accounts for approximately 70% of prostanoid release from buffer-perfused rat hearts [de Deckere *et al.*, 1977]. PGE_2 contributes about 15%, PGD_2 and $PGF_{2\alpha}$ less than 10%. A small amount of TXA_2 is released from perfused hearts, perhaps from adherent platelets. In blood vessels, PGI_2 is released in amounts

approximately 5- and 10-fold greater than $PGF_{2\alpha}$ and PGE_2, respectively [Moncada and Vane, 1979, Pipili and Poyser, 1981].

1.2.6 Specialised rôles of phospholipids: cell signalling

A variety of hormones and transmitters, including α_1-adrenoceptor agonists, evoke a rise in cytosolic Ca^{2+} levels. It is now widely accepted that PI is an important link in receptor-mediated mobilisation of intracellular Ca^{2+} stores [Michell, 1975]. PI is unusual in that it exists in two more phosphorylated forms. These are phosphatidylinositol-4-monophosphate and phosphatidylinositol-4,5-*bis*-phosphate; PIP and PIP_2 respectively. PIP and PIP_2 constitute less than 2% of total cell inositol phospholipid but this proportion may be 10-fold higher in cell membranes [Augert *et al.*, 1989]. Stimulation of the α_1-adrenoceptor, for example, activates membrane-bound phospholipase C which hydrolyses PIP_2 to release inositol-1,4,5-*tris*-phosphate (IP_3) and diacylglycerol (DAG) [Berridge, 1983]. IP_3 functions as a cytosolic second messenger for the release of intracellular Ca^{2+} [Berridge and Irvine, 1984] and DAG operates within the plane of the cell membrane to activate protein kinase C [Nishizuka, 1984].

Recognition of the pivotal rôle of inositol phospholipids in cell signalling has encouraged other lines of investigation. Recent reports suggest that the more abundant phospholipid PC may be another source of second messengers. In response to diverse agonists, phospholipase C can release DAG from PC [Exton, 1990]. It is also possible that phosphatidic acid, derived from PC by the actions of phospholipase D, may be an alternative second messenger involved in sustained Ca^{2+} release [Ohanian *et al.*, 1990].

The following sections relate to α_1-adrenoceptors in blood vessels and the heart and the exciting topic of phospholipid-dependent signal transduction will be further discussed.

1.3 The α_1-adrenoceptor in the cardiovascular system

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1.3.1 History and classification of α-adrenoceptors

Establishing the principle that receptors should be characterised by their capacity to recognise and respond to certain agents, rather than according to their site and the tissue response elicited, Ahlquist [1948] proposed that adrenoceptors be divided into two separate categories; α and β. In a similar way, the different susceptibilities of α-adrenoceptor populations in the cat spleen to phenoxybenzamine led to the concept of post-junctional α_1-adrenoceptors mediating contraction, and pre-junctional α_2-adrenoceptors exerting negative feedback on neuronal noradrenaline release [Langer, 1974]. However, it has since become clear that the α_2-adrenoceptor exists beyond the confines of the sympathetic neuromuscular junction, at post-junctional sites where it activates smooth muscle cell contraction [Drew and Whiting, 1982, Gardiner and Peters, 1982, McGrath, 1982]. Indeed, the α_2-adrenoceptor may be more important than the α_1-adrenoceptor in vasoconstriction at the level of the pre-capillary arteriole [Faber, 1988]. There is also evidence to suggest a pre-junctional, negative feedback regulatory rôle for the α_1-adrenoceptor [Kobinger and Pichler, 1982, Docherty, 1983, Story *et al.*, 1985]. Thus, we have pre- and post-junctional α_1-adrenoceptors, and pre- and post-junctional α_2-adrenoceptors.

The α_1- and α_2-adrenoceptors are further distinguished by their signal transduction mechanisms. Generally, signalling for the α_1-adrenoceptor involves the breakdown of inositol phospholipids, mentioned above (section 1.2.6) and discussed further below, whereas α_2-adrenoceptor activation inhibits adenylate cyclase and decreases intracellular cyclic AMP levels [Fain and Garcia-Sainz, 1980].

On the basis of heterogeneous affinity for imidazolines and phenylethylamines, the uniform α_1-adrenoceptor classification has been questioned. It is proposed that α_{1a}-receptors; activated by imidazolines and low concentrations of phenylethylamines and often sensitive to Ca^{2+} antagonists, operate by opening Ca^{2+} channels. In contrast, α_{1b}-receptors; activated by high concentrations of phenylethylamines,

provoke the inositol phospholipid-dependent release of intracellular Ca^{2+} stores. The topic of α_1-adrenoceptor sub-classification, still a source of debate, is thoroughly reviewed by Minneman [1988]. An alternative explanation, in view of many exceptions to the α_{1a}/α_{1b} definitions, is that α_1-adrenoceptors share a common recognition site but have diverse agonist affinities [Bevan *et al.*, 1988] or perhaps signalling mechanisms [McGrath and Wilson, 1988].

1.3.2 α_1-adrenergic signal transmission

The α_1-adrenoceptor is an integral membrane protein, or glycoprotein, comprising a single polypeptide chain of approximately 80 kDaltons [Leeb-Lundberg *et al.*, 1984]. The receptor is coupled to a toxin-insensitive G protein (GTP-binding protein) which may be G_q, a 42 kDalton protein [Pappano *et al.*, 1988, Pang and Sternweis, 1990]. G proteins are heterotrimers, consisting of α, β and γ subunits. Following agonist-receptor binding, GTP is exchanged for the GDP attached to the inactive α subunit and the β/γ subunits together dissociate from the G protein complex. The now active α subunit in turn activates membrane-bound phospholipase C. During deactivation of the G protein, the intrinsic GTPase activity of the α subunit restores α-GTP to α-GDP which reassociates with the β/γ subunits. Current concepts of G proteins have been reviewed recently [Holmer and Homcy, 1991, Fleming *et al.*, 1992].

Phospholipase C releases IP_3 and DAG from PIP_2 as described above (section 1.2.6) and PIP_2 hydrolysis in relation to α_1-adrenoceptor stimulation has been demonstrated in the rat aorta and heart [Jenkin *et al.*, 1991, Otani *et al.*, 1988]. IP_3 mobilises intracellular Ca^{2+} [Berridge and Irvine, 1984] which can exert small direct positive inotropic effects in the myocardium [Otani *et al.*, 1988], and influence trans-membrane ion flux such as Na^+/Ca^{2+} exchange [Gilbert *et al.*, 1991]. IP_3 can be phosphorylated to inositol-1,3,4,5-*tetrakis*-phosphate, another putative intracellular second messenger. Inositol is regenerated for fresh PI synthesis by a series of phosphatases. Two of these phosphatases are inhibited by Li^+, inositol polyphosphate-1-phosphatase and inositol monophosphatase. The phosphoinositide cycle has been succinctly summarised by Nahorski *et al.* [1991].

The rôle of DAG is twofold; it is an activator of protein kinase C and, through the actions of phospholipase A_2, a source of 20:4, n-6 [Berridge, 1984, Nishizuka, 1986]. It is possible that unsaturated fatty acids, such as 20:4, n-6, can also activate protein kinase C [McPhail *et al.*, 1984, Murakami and Routtenberg, 1985]. Protein kinase C is a phospholipid- (PS) dependent enzyme [Nishizuka, 1983, Nishizuka, 1984] which is thought to travel in a Ca^{2+}-dependent manner from the cytosol to the cell membrane at the time of activation [Khalil and Morgan, 1991]. Once activated, protein kinase C is involved in the phosphorylation of regulatory proteins, likely to be a mechanism in α_1-adrenergic effects on the slow inward Ca^{2+} current [Lindemann, 1986], on Na^+/H^+ exchange [Otani *et al.*, 1990] and cardiac contractility [Talosi and Kranias, 1992]. Protein kinase C may also render the α_1-adrenoceptor inactive by phosphorylation, thus exerting a negative feedback effect [Lefkowitz and Caron, 1986].

1.3.3 Aspects of vascular α_1-adrenoceptor function

Co-transmission and synergism in α_1-adrenergic effects continue to attract a great deal of interest, partly because of therapeutic implications. Work with rat and guinea-pig vas deferens preparations first suggested that neuronal noradrenaline release might be associated with the release of another transmitter [Blakely *et al.*, 1981] This putative second transmitter was identified as ATP, which occupies a specific purinergic receptor (P_{2X}) on the smooth muscle cell and elicits excitatory junction potentials [Sneddon and Burnstock, 1984]. ATP co-transmission was subsequently demonstrated in the isolated rabbit saphenous artery [Burnstock and Warland, 1987]. As a consequence of the ATP-induced excitatory potentials, dihydropyridine-sensitive slow Ca^{2+} channels are activated, explaining the finding of Blakely *et al.* [1981] that a component of the vas deferens response was sensitive to nifedipine. This effect of dihydropyridines has also been shown for α_1-adrenoceptor responses to exogenous noradrenaline [Dunn *et al.*, 1991*c*].

The synergistic relationship of angiotensin II with adrenergic responses was uncovered by the effects of captopril, an angiotensin converting enzyme (ACE)

inhibitor, which reduced responses to sympathetic nerve stimulation and to α_1-adrenoceptor agonists in pithed rats [Hatton and Clough, 1982, Richer *et al.*, 1984]. The effect was abolished by prior nephrectomy. The relationship between angiotensin II and sympathetically mediated haemodynamic responses in the pithed rat was further investigated by Kaufmann and Vollmer [1985]. These authors distinguished the actions of angiotensin II on peripheral vascular resistance in the context of endogenous and exogenous sympathetic stimulation from those in unstimulated preparations, where angiotensin II appears to support BP by maintaining cardiac output, probably through a reduction in venous capacitance. Recent evidence suggests that the facilitatory effects of angiotensin II, whether in relation to sympathetic nerve stimulation or the application of exogenous agonist, may be relatively selective for the α_2- rather than the α_1-adrenoceptor [Dunn *et al.*, 1991*a*, Dunn *et al.*, 1991*b*].

The existence of post-junctional α_2-adrenoceptors capable of causing vasoconstriction has provoked much research into the rôles of α_1- and α_2-adrenoceptors in vasoregulation. The greater effects of prazosin on pressor responses to nerve stimulation, as opposed to exogenous noradrenaline, gave rise to the concept that α_1-adrenoceptors were innervated, whereas α_2-adrenoceptors were not [Langer *et al.*, 1980, Docherty and McGrath, 1980, Langer *et al.*, 1981]. In pithed rats injected with DMPP (1,1-dimethyl-4-phenylpiperazine), which releases catecholamines from sympathetic neurones and the adrenal medulla, bilateral adrenalectomy abolished pressor responses mediated by the α_2-adrenoceptor [Wilffert *et al.*, 1982]. The authors concluded that circulating adrenaline, rather than neuronal noradrenaline, was the normal source of α_2-adrenoceptor stimulation. However, against these findings, α_2-adrenoceptor antagonists augmented the effect of prazosin on pressor responses to nerve stimulation in perfused hind limb preparations [Madjar *et al.*, 1980, Gardiner and Peters, 1982, Elsner *et al.*, 1984, Hamed *et al.*, 1986]. Interestingly, reflex vascular responses to exercise and cold were preserved in men given sufficient prazosin to lower BP [Mancia *et al.*, 1980], suggesting that α_2-adrenoceptors have some function

in sympathetic autoregulation. The relative contribution of α_1- and α_2-adrenoceptors to resting vascular tone is more difficult to ascertain. Although the effects of α_1-adrenoceptor antagonists are readily interpreted, those of α_2-adrenoceptor antagonists are usually complicated by their pre-synaptic actions. Nevertheless, the α_2-adrenoceptor antagonist yohimbine has been reported to cause a dose-dependent increase in resting forearm blood flow in normal volunteers [van Brummelen *et al.*, 1983].

In attempts to establish the physiological rôles of α_1- and α_2-adrenoceptors, it has been proposed that their distribution is heterogeneous. In canine coronary arteries, the proximal (conduit) vessels and distal (resistance) vessels appeared to be subserved respectively by α_1- and α_2-adrenoceptors [Heusch *et al.*, 1984] with the dominant effect on coronary vasoconstriction being mediated by α_2-adrenoceptors [Da Huang Chen *et al.*, 1988]. However, others have found that α_1- and α_2-adrenoceptors exert equivalent effects in the coronary vascular bed [Woodman and Vatner, 1987, Miyamoto *et al.*, 1991]. In skeletal muscle, adrenergic effects on large arterioles and venules were mediated by α_1- and α_2-adrenoceptors, but α_2-adrenoceptors were more important in pre-capillary arterioles [Faber, 1988]. Experimental conditions are important in these studies as environmental effects on α-adrenoceptors must be considered. McGrath *et al.* [1982] demonstrated in the pithed rat that alkalosis favours α_1-adrenoceptor activation and acidosis, α_2-adrenoceptor activation. Consistent with this observation, sympathetic vasoconstriction distal to a severe coronary stenosis was dependent on α_2-adrenoceptors [Heusch and Deussen, 1983] and coronary hypoperfusion was necessary to demonstrate an effect of the α_2-adrenoceptor agonist BHT-933 on vessels less than 100 μm in diameter [Chilian, 1991]. In contrast, low flow and metabolic acidosis diminished constrictor responses to α_2-adrenoceptor stimulation in rat arterioles [McGillivray-Anderson and Faber, 1991, Anderson and Faber, 1991]. The influence of altered regional flow and acid-base balance on α-adrenoceptor function remains incompletely understood.

Other factors may affect the α-adrenoceptor. Cooling suppresses α_1-adrenoceptor responses and enhances α_2-adrenoceptor responses [Freedman *et al.*, 1992], similar effects to age [Nielsen *et al.*, 1992]. Oestrogens are associated with greater α-adrenoceptor affinity for noradrenaline and adrenaline [Colucci *et al.*, 1982]. In dogs, pacing-induced heart failure was found to enhance peripheral vascular α_1-adrenoceptor responses [Forster and Armstrong, 1992]. Curiously, in this last study, there was no investigation of the likely contribution of elevated renin and angiotensin levels to the observed effects.

Effects of α_1-adrenoceptor stimulation *in vivo* are readily demonstrated *in vitro*. This is not so for the α_2-adrenoceptor. The difficulty in eliciting responses to selective α_2-adrenoceptor agonists in isolated vessels has led authors to conclude that various arterial and venous preparations are devoid of functional α_2-adrenoceptors [Langer and Hicks, 1984, Nielsen *et al.*, 1991]. These conclusions may be erroneous. Dunn *et al.* [1991*c*] (referred to above) have elegantly demonstrated that α_2-adrenoceptor responses can be revealed by, and may indeed be dependent upon, a degree of vascular smooth muscle stimulation conferred by angiotensin II or α_1-adrenoceptor activation.

1.3.4 Aspects of cardiac α_1-adrenoceptor function

Controversy regarding the cardiac α-adrenoceptor has been sufficient to provoke the question, "Are there α-adrenoceptors in the mammalian heart?" [Schümann, 1980]. In fact , inotropic actions of phenylephrine (an α_1-adrenoceptor agonist), which were abolished by phentolamine (an α-adrenoceptor antagonist), were demonstrated in rat ventricular strips nearly thirty years ago [Wenzel and Su, 1966]. It has since been shown that responses of the rat heart to the physiological agonists noradrenaline and adrenaline are readily unmasked by β-adrenoceptor blockade [Ask and Stene-Larsen, 1984]. There are important species differences in cardiac α_1-adrenoceptor density [Steinfath *et al.*, 1992] in that the rat has a density 5 to 8-fold greater than the guinea-pig, rabbit or man, for example. Although the presence and function of α_2-adrenoceptors is easily shown in the coronary arteries (see previous section), this adrenoceptor subtype seems to be almost non-existent in the myocardium [Williams *et*

al., 1981]. The physiological rôle of myocardial α_1-adrenoceptors remains uncertain but their function in pathophysiological circumstances, such as myocardial ischaemia and heart failure, has attracted considerable attention. Broadly speaking, research efforts have followed three directions; first, the behaviour of the α_1-adrenoceptor during and after myocardial ischaemia, second, α_1-adrenoceptor effects on cardiac contractility and, third, the effects of α_1-adrenoceptor stimulation and blockade on cardiac dysrhythmogenesis.

Approximately 30 minutes following coronary artery ligation *in vivo*, an increase in cardiac α_1-adrenoceptor density, assessed by radio-ligand binding, has been observed in the dog [Mukherjee *et al.*, 1980], the cat [Corr *et al.*, 1981], the guinea-pig [Maisel *et al.*, 1986] and the rat [Allely and Brown, 1988]. It has been suggested that, in the rat, this is a reflex phenomenon dependent upon intact autonomic innervation [Dillon *et al.*, 1988]. However, an increase in α_1-adrenoceptor density has also been shown in isolated rat cardiomyocytes subjected to temporary hypoxia [Matsuo *et al.*, 1986]. Evidence from isolated canine cardiomyocytes and rat hearts *in vivo* suggests that the ischaemia-induced increase in α_1-adrenoceptor density may be dependent upon long-chain acyl carnitines [Heathers *et al.*, 1987, Allely and Brown, 1988]. Regional ischaemia has also been associated with enhanced chronotropic responses to the α_1-agonist methoxamine [Sheridan *et al.*, 1980].

As a consequence of downregulation, the β_1-adrenoceptor reserve declines in heart failure [Brown *et al.*, 1992] and cardiac responses to β_1-adrenoceptor stimulation may be further impaired by G_S protein dysfunction [Longabaugh *et al.*, 1988, Kessler *et al.*, 1989, Feldman *et al.*, 1990]. The α_1-adrenoceptor does not appear to be similarly affected. In the cardiomyocytes of genetically cardiomyopathic hamsters [Sen *et al.*, 1990], and in ventricular tissue from patients suffering severe heart failure (NYHA Grade IV) due to idiopathic dilated cardiomyopathy [Böhm *et al.*, 1988], the density of α_1-adrenoceptors, reflected by ^{3}H-prazosin binding sites, was preserved and responses to α_1-adrenoceptor stimulation maintained or enhanced. There were similar findings with respect to adrenoceptor density in relation to supraphysiological pacing-induced

failure in healthy dogs, but responses to both α_1- and β_1-adrenoceptor stimulation decreased [Calderone *et al.*, 1991]. Diabetic cardiomyopathy induced by streptozotocin in rats was associated with a decrease in both ^{3}H-dihydroergocriptine and ^{3}H-dihydroalprenolol binding sites [Heyliger *et al.*, 1982], but inotropic responses to methoxamine in the presence of practolol were found increased in lambs with alloxan-induced diabetes mellitus [Downing *et al.*, 1983].

Although there is considerable interest in the apparent susceptibility of the adrenoceptors, particularly the β-adrenoceptor, to these diverse pathophysiological states, the important effects of increased circulating catecholamines, associated with adrenoceptor down-regulation, should not be overlooked. The elevated levels of noradrenaline and adrenaline found in heart failure would also be expected in relation to myocardial ischaemia *in vivo* and experimental diabetes. Further, it is possible that enhanced α_1-adrenoceptor reponsiveness reflects a synergistic effect of angiotensin II (referred to in section 1.3.3), levels of which are likely to be increased in the above conditions.

The maintenance of myocardial α_1-adrenoceptor function, in circumstances adversely affecting the β_1-adrenoceptor, suggests that the α_1-adrenoceptor is part of a reserve mechanism when β_1-adrenoceptor function is perturbed. Aside from beneficial effects on contractility, α_1-adrenoceptor stimulation may confer metabolic advantages. It was proposed recently that the protective effect of α_1-adrenoceptor stimulation in the context of myocardial stunning was in part due to the increased release and availability of adenosine [Kitazake *et al.*, 1991]. Unfortunately, peripheral and pulmonary arterial and venous effects of α_1-adrenoceptor stimulation tend to have adverse effects on cardiac preload and afterload [Leier *et al.*, 1990].

The positive inotropic effects of α_1- and β_1-adrenoceptor stimulation differ. In isolated papillary muscles, selective α_1-adrenoceptor stimulation lengthens the action potential, thus increasing and delaying peak tension, and prolonging the duration of contraction [Dukes and Vaughan Williams, 1984, Bruckner *et al.*, 1984]. This is quite distinct from the characteristic β_1-adrenoceptor effects of accelerating both the

development and relaxation of tension. As a corollary, α_1-adrenoceptor stimulation has been shown to have minimal effects on $LVdP.dt^{-1}$ in the perfused heart [Aoyagi *et al.*, 1991]. Indeed, the effects on $LVdP.dt^{-1}$ in this study may be partly explained by residual β_1-adrenoceptor stimulation; up to 10 μM phenylephrine was used in the presence of 1 μM propranolol. It is curious that studies in different species have nevertheless used $LVdP.dt^{-1}_{max}$ as the preferred end-point of α_1-adrenoceptor stimulation [Downing *et al.*, 1983, Reibel *et al.*, 1988].

It was quickly appreciated that the effect of α_1-adrenoceptor stimulation was independent of cyclic AMP (adenosine-3',5'-monophosphate) [Osnes *et al.*, 1973, Osnes, 1976, Scholz, 1980], and unaffected or enhanced by adenosine [Endoh and Yamashito, 1980] and acetylcholine [Inui *et al.*, 1982], both of which diminish cyclic AMP-mediated effects. Recognition of the rôles of IP_3 and DAG in cell signalling (discussed above) was followed by the demonstration of PIP_2 hydrolysis following α_1-adrenoceptor stimulation in cardiomyocytes [Brown *et al.*, 1985]. Others have since reported that IP_3 is responsible for the initial phases of the inotropic response, and DAG-activated protein kinase C for the sustained response [Otani *et al.*, 1988, Otani *et al.*, 1990]. Indeed, although IP_3 generation (measured by IP_1 accumulation) approximately parallelled the inotropic response to epinephrine in the presence of β-adrenoceptor blockade in the rat, this was not so in the rabbit, suggesting that this product of PIP_2 hydrolysis does not account for all the effects of α_1-adrenoceptor stimulation [Endoh *et al.*, 1991]. Recent studies using inhibitors of Na^+/H^+ exchange, such as hexamethylamiloride, demonstrate that these agents can partly abolish the inotropic effects of α_1-adrenoceptor stimulation [Otani *et al.*, 1990, Terzic and Vogel, 1991]. Activation of Na^+/H^+ exchange, possibly by protein kinase C, raises intracellular pH which facilitates myofibrillar Ca^{2+} binding [Blanchard and Solaro, 1984]; presumably the explanation for the finding of Endoh and Blinks [1988] that α_1-adrenoceptor stimulation was linked to changes in myofibrillar Ca^{2+} responsiveness. Another putative mechanism in the positive inotropic effects of α_1-adrenoceptor stimulation has been highlighted by Fedida and Bouchard [1992]. They showed that in

rat cardiomyocytes, the inward Ca^{2+} current was sustained, but not increased, by prolongation of the action potential in association with α_1-adrenoceptor stimulation. The prolongation of the action potential was demonstrated to be due to a decrease in outward K^+ current, an effect previously attributed to the α_1-adrenoceptor [Apkon and Nerbonne, 1988, Ravens *et al.*, 1989, Fedida *et al.*, 1990]. Detailed electrophysiological studies such as these are necessary to unravel the complex cellular effects of α_1-adrenoceptor stimulation.

In addition to the effects of the α_1-adrenoceptor on cardiac contractility are effects on cardiac rhythm and dysrhythmogenesis. The chronotropic actions of α_1-adrenoceptor stimulation are complex and remain a source of confusion. In isolated human and rat atria, a negative chronotropic effect was associated with, respectively, delayed phase IV depolarization and delayed repolarization [Mary-Rabine *et al.*, 1978, Dukes and Vaughan Williams, 1984]. Conversely, studies in the pithed rat revealed a positive chronotropic effect of both exogenous and endogenous catecholamines [Flavahan and McGrath, 1981]. In a subsequent study, these authors uncovered biphasic effects of α_1-adrenoceptor stimulation on heart rate in the same preparation; a transient negative chronotropic effect was detected within the overall positive chronotropic effect. The negative chronotropic effect was removed by atropine and hexamethonium, and enhanced by neostigmine, indicating that a positive feedback effect on parasympathetic nerves at a pre-ganglionic level had taken place. It has also been claimed that α_1-adrenoceptor stimulation exerts a negative feedback effect on parasympathetic outflow; phenylephrine antagonised the bradycardic effects of vagal stimulation in rats [McGrattan *et al.*, 1987]. However, the latter study did not exclude the likely effects of this particular agonist on post-junctional α_1- and β_1-adrenoceptors. There is evidence that the incidence of ventricular dysrhythmia precipitated by myocardial ischaemia may be reduced by α_1-adrenoceptor antagonists, and increased by α_1-adrenoceptor agonists. Prazosin was protective against reperfusion ventricular fibrillation induced by regional ischaemia in cats and rats [Sheridan *et al.*, 1980, Thandroyen *et al.*, 1983]. Also, prazosin reduced the incidence

of ventricular fibrillation in response to post-infarction ischaemia in dogs [Wilber *et al.*, 1987]. In the study of Sheridan *et al.* [1980], a secondary finding was that the adverse effects of left stellate ganglion stimulation during reperfusion could be inhibited by phentolamine, and partly reproduced by intracoronary methoxamine. Following chemical denervation with 6-hydroxydopamine, methoxamine increased the incidence of ischaemic and reperfusion ventricular tachycardia and fibrillation in perfused guinea-pig hearts [Culling *et al.*, 1987]. Difficult to dissect from the results in studies of this nature is whether the favourable effect of α_1-adrenoceptor antagonism is due to an electrophysiological effect or to a beneficial redistribution of coronary flow. Although cæsium-induced afterdepolarizations and ventricular dysrhythmias were aggravated by phenylephrine (in the presence of propranolol) and alleviated by prazosin in dogs [Ben-David and Zipes, 1990], another recent study found both pro- and anti-arrhythmic effects of α_1-adrenoceptor stimulation on oscillatory afterpotentials and triggered automaticity in isolated rabbit Purkinje tissue, depending on the means of induction (strophanthin or high $[Ca^{2+}]_o$) [Han and Ferrier, 1990]. Thus, pure electrophysiological studies are equally difficult to interpret. It has been suggested that contrasting effects of traditional α_1-adrenoceptor agonists on dysrhythmogenesis might be explained by the presence in conducting tissue of α_{1a}- and α_{1b}-adrenoceptors, respectively increasing and decreasing automaticity [del Balzo *et al.*, 1990, Molina-Viamonte *et al.*, 1991] but it is probably unwise to assume that this subclassification is widely applicable, as was alluded to above (section 1.3.1). For a helpful overview of the general rôle of α-adrenoceptors in arrhythmogenesis, the reader may refer to McGrath [1989].

The final sections of this Introduction deal with established and proposed links between PUFA and adrenergic events.

1.4 Interactions: PUFA and adrenergic mechanisms

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Several of the reports cited in previous sections either reveal or imply effects of PUFA on adrenergic mechanisms. These are briefly highlighted together with other relevant literature.

1.4.1 Dietary PUFA and adrenoceptor responses

Studies performed in isolated rat aortic tissue have consistently shown that dietary n-3 PUFA are associated with attenuated responses to exogenous noradrenaline [Lockette *et al.*, 1982, Yin *et al.*, 1991, Malis *et al.*, 1991]. In the isolated rat heart also, inotropic responses to selective α_1-adrenoceptor stimulation were reduced by n-3 PUFA [Reibel *et al.*, 1988]. These results imply an effect of n-3 PUFA on the α_1-adrenoceptor or on the intracellular events which follow α_1-adrenoceptor stimulation. In contrast to these data, the effects of dietary PUFA, commonly n-6 PUFA, on the responses of cardiac tissue to β_1-adrenoceptor stimulation vary widely. Enhancement [Hoffman *et al.*, 1982, Charnock *et al.*, 1987], attenuation [Wince and Rutledge, 1981, Wince *et al.*, 1987] and a lack of effect [Reibel *et al*, 1988] of diet on β_1-adrenoceptor responses have all been demonstrated. These inconsistencies raise the possibility that dietary effects on adrenoceptors may be relatively selective for the α_1-adrenoceptor.

Noticeable in the above studies is the lack of information regarding the effects of the various experimental diets on tissue fatty acid composition; only Lockette *et al.* [1982] and Reibel *et al.* [1988] provide such data and it is limited to total phospholipid fatty acid composition.

1.4.2 Dietary PUFA and the regulation of noradrenaline release

There is much evidence for the rôle of certain prostanoids in the control of neuronal noradrenaline release. PGE_2 appears to exert a negative feedback effect while $PGF_{2\alpha}$ may do the opposite [Hedqvist, 1977]. The ability of dietary n-3 PUFA to alter the balance of dienoic and trienoic prostanoids may thus have consequences for sympathetic regulation. PGI_2, the principal prostanoid released by vascular and cardiac

tissues, does not appear to have any pre-junctional effects [Hedqvist, 1977, Armstrong and Thirsk, 1979].

The group of Dixon and co-workers has performed a series of investigations examining the effect of diet on tissue noradrenaline content and release, focusing on n-6 PUFA *versus* saturated fatty acids. In relation to n-6 PUFA, they repeatedly show a decrease in noradrenaline release measured during field stimulation in isolated rat tail [Panek *et al.*, 1985, Semafuko *et al.*, 1989] and heart [Semafuko *et al.*, 1987] preparations. The results for tissue noradrenaline content were less consistent, being increased in the first report, unchanged in the second, and decreased in the third, following dietary n-6 PUFA. Although the same experimental diets were administered in each of these studies, the authors offer no data regarding the effects of these diets on tissue fatty acid composition. Such data might aid interpretation of the variable effects of diet on noradrenaline content. It is difficult to marry these results with those of dietary intervention studies in animals where n-6 PUFA are generally associated with a fall in BP (see section 1.1.3).

Pressor responses to various forms of stress in rats (see section 1.1.5) and man (see section 1.1.4) have been blunted by both n-3 PUFA [Mills and Ward, 1986, Singer *et al.*, 1985, di Giacomo *et al.*, 1989] and n-6 PUFA [Butcher *et al.*, 1990]. These interesting findings reflect reduced sympathetic activation and are obviously consistent with a number of mechanisms, including neuronal and central effects.

1.4.3 Dietary PUFA, signalling and Ca^{2+} channels.

Dietary PUFA affect the fatty acid composition of cell membrane phospholipids, principally by altering the nature of the fatty acid species esterified at the *sn*-2 position, where n-3 and n-6 series PUFA compete (see section 1.2.4). Increased incorporation of unsaturated fatty acids renders the cell membrane more fluid and this is thought to influence the activity of enzymes associated with the membrane, such as protein kinase C [Epand and Lester, 1990]. Phospholipase A_2 activity has been altered by PUFA in the diet [Malis *et al.*, 1990] and in cell culture medium [Nalbone *et al.*, 1990].

The conformational changes brought about by the presence of longer-chain unsaturated fatty acid species in PI might be expected to have implications for the generation of intracellular second messengers from PIP_2 (see section 1.2.6). This has been shown to be the case. In a variety of studies, n-3 PUFA in the diet [Medini *et al.*, 1990], and in cell culture media [Locher *et al.*, 1988, Bordoni *et al.*, 1990], have attenuated the synthesis or release of IP_3. Evidence for direct effects of n-6 PUFA on adrenoceptors and signalling is more limited, but reductions in β-adrenoceptor density and affinity, and adenylate cyclase activity, were found in the isolated atria of rats fed an n-6 PUFA diet [Wince and Rutledge, 1981].

Cell membrane ion channels may also be influenced by dietary PUFA. In a series of investigations, dietary n-6 and n-3 PUFA attenuated the responses of rat papillary muscles to external Ca^{2+} [Charnock *et al.*, 1985, McLennan *et al.*, 1987]. However, the same was not true of dietary n-6 PUFA in the marmoset [McLennan *et al.*, 1987*b*]. Unfortunately, none of these studies provides data on tissue fatty acid composition which would have been useful in considering further the mechanism(s) underlying the observations.

1.5 Hypothesis

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Dietary PUFA reduce vascular and cardiac responses to α_1-adrenoceptor stimulation by altering the fatty acid composition of cell membrane phospholipids.

Chapter 2

Methods

2.1 Experimental animals

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Throughout the project, weaned male Sprague-Dawley rats (Banting and Kingman, The Field Station, Grimston, Hull, England) or weaned male Lew rats (Harland Olac, Shaws Farm, Blackthorn, Bicester, Oxfordshire, England) were used. For dietary studies, rats six weeks of age were obtained. For smaller scale experiments performed in order to define aspects of methodology, rats were purchased by weight at 300-400 g, typical of the final weight achieved with the experimental diets. Animals were housed, no more than four in a cage, under standard conditions of a twelve hour light/dark cycle and ambient temperatures of 20-21° C. Routinely, cages were changed three times a week and food and water were allowed *ad libitum*. Body weights were recorded weekly.

2.2 Experimental diets

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2.2.1 Ingredients of experimental diets

Semisynthetic experimental diets were used throughout the project. These were prepared from the basic ingredients of casein, cellulose, cornflour, edible fats and oils, salt mixture and vitamin mixture. Diets compared in individual experiments were designed to be isocalorific. The diet most used as a control derived 40% of total dietary calories from fat, 37% from carbohydrate and 23% from protein. Low fat diets

obtaining 10% of total calories from fat had the contribution from carbohydrate increased appropriately (Appendix 1). The required balance of fatty acids was obtained by using different edible fats and oils (beef dripping, olive oil, safflower oil and sunflower oil) of which the fatty acid composition had been determined. Published food tables [McCance and Widdowson, 1978] were used to estimate the amounts of vitamin E provided by these sources. Different experimental diets were rendered equivalent in vitamin E content (80 $mg.kg^{-1}$) by the addition of *d*-α-tocopherol acetate as required. The quantities of salts and vitamins appropriate to the diets were derived from the work of Unilever Research, Vlaardingen, The Netherlands.

2.2.2 Preparation and storage of experimental diets

Drystuffs for the diets, including the bulk salt and vitamin mixes, and complete diet mixtures were prepared in the departmental diet kitchen using a "Robot Coupe" commercial food mixer (capacity 2-3 kg). Dry ingredients, casein, cellulose and cornflour, were combined as batches sufficient for 2 kg of complete diet and stored in sealed polythene bags at room temperature. Fat mixtures (Appendix 2) were made by combining oils with melted solid fats so as to be sufficient for several 2 kg quantities of complete diet. Vitamin E, if required, was added to the fat mixture which was then stored in sealed plastic tubs for less than two weeks at -10° C. Dietary salt components (Appendix 3) were combined and mixed for 2-5 minutes in the Robot Coupe. Thereafter, 46 g lots of salt mixture, sufficient for 2 kg of complete diet, were dispensed into sealed plastic cups before being stored at room temperature. Fresh vitamin mix (Appendix 4) was prepared as required and 8 g quantities were weighed into screw-top plastic vials, kept at -10° C.

Complete diets were prepared weekly by blending together the above classes of ingredient in the Robot Coupe. Drystuffs, salts and vitamins were combined at slow speed and the correct volume of molten fat mix gradually poured in. The ingredients

were then mixed at high speed for 2 minutes. Water was not added and the food was not pelleted. To safeguard against peroxidation, diets were stored under argon in sealed polythene containers at -10° C. Diets containing large proportions of fish oil (Chapter 5) were assumed to be more prone to peroxidation and were therefore prepared freshly every 48 hours. Half was used immediately and the remainder kept at -10° C, under argon, in glass jars sealed with screw-on lids and clingfilm, for less than 24 hours.

2.2.3 Feeding of experimental diets

Fresh supplies of experimental control and n-6 PUFA diet were provided thrice weekly when the animal cages were changed. When the diets containing fish oil were in use, these were provided daily and cages were also changed daily to ensure the removal of spilled diet at risk of peroxidation.

2.2.4 Preparation and administration of dietary supplements

Certain experiments (Chapters 3, 4, 6 and 7) required the use of small amounts of n-3 PUFA which added only 0.4% to the total dietary calories i.e. less than 0.1 ml fish oil per day. Volumes of this order could not be mixed into a complete diet, the dilution being too great to allow even distribution. The sole means of ensuring the consumption of these volumes of fish oil, and of being certain that there was no opportunity for peroxidation, was oral administration. Accordingly, rats were dosed transoesophageally with fish oil diluted in olive oil (total volume 0.5-0.7 ml) using a short plastic cannula (initially an Argyle® 5G Infant Feeding Catheter, later a similar device fashioned from a 16G Medicut® Intravenous cannula and the tip of an Argyle® 6G Infant Endotracheal Suction Catheter, all Sherwood Medical, Tullamore, Eire). The olive oil used to dilute the fish oil was not supplementary as it had been withheld from the original olive oil content of the diet. Similarly, olive oil withheld from the fat content of the control and n-6 PUFA diets provided control oral doses, to which fish

oil was not added, for these other animals. The mixtures of fish oil and olive oil were prepared in advance, placed in 20 ml universal containers under argon and kept at -20° C for less than one week until the day of use, being allowed to thaw just prior to administration. A fresh container was used each day and any excess was discarded. Olive oil for control doses, at less risk of peroxidation, was simply refrigerated in sealed containers.

2.2.5 Monitoring the effect of experimental diets: tissue fatty acid composition

To assess the consumption of the n-6 PUFA experimental diets, adipose tissue was sampled for fatty acid analysis as part of the surgical procedures of the various experiments. Approximately 1 g perinephric adipose tissue was placed in an Eppendorff tube and stored at -40° C pending assay. The influence of the diets on the fatty acid composition of total and fractionated phospholipid was assessed using cardiac tissue. Where the experiments concerned vascular tissue (Chapters 3 and 4), analysis of the fatty acid composition of the target tissue could not be performed due to the amount of tissue required. Hearts were excised carefully after the commencement of hind-quarters perfusions (Chapter 3), before isolated resistance artery studies (Chapter 4) or retained after isolated heart perfusions (Chapters 5,6 and 7). The hearts were trimmed of atria and great vessels, opened, rinsed free of blood in ice-cold buffer if necessary, blotted dry and placed in Nunc tubes stored at -70° C pending assay.

2.3 Preparation of drugs

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For all experiments, solutions of drugs were prepared freshly on the day of administration. During a series of experiments, agents in frequent use, e.g. noradrenaline and timolol, were first prepared as concentrated stock solutions

(1-5 mM in 0.01 M HCl) and refrigerated in dark brown semi-opaque flasks for less than five days. Fresh solutions of noradrenaline for infusion (thus diluted a final 1:30-60 on infusion) were made every 3-4 hours during an experimental day, contained ascorbic acid 1 $mg.ml^{-1}$ and were kept in the dark on ice.

In one experiment, 6-hydroxy-dopamine was required (section 2.6.6 and Chapter 5). The chloride salt was kept frozen with dessicant at -20° C and the appropriate drug concentration, in sterile 0.9% saline containing 1 $mg.ml^{-1}$ ascorbic acid, was only prepared immediately prior to dosage.

2.4 Hind-quarters perfusion studies

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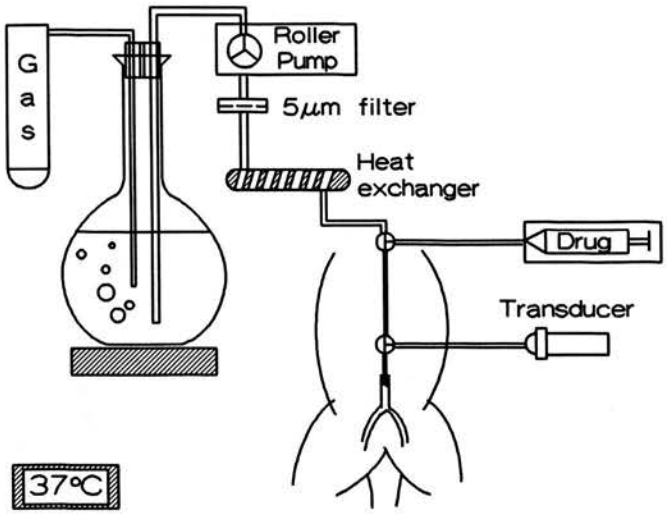
2.4.1 Perfusion apparatus

The method for the perfusion of the rat hind-quarters was modified from Folkow *et al.* [1970]. The apparatus used for these experiments is displayed diagrammatically in Figure 2.1. Gassed buffer warmed to 37° C was drawn through gas-impermeable tubing from a 5 L flask by a Watson-Marlow roller pump and fed through rigid manometer tubing incorporating a 5 μm filter to a glass heat exchanger. A further short length of manometer tubing carried buffer to a grooved perfusion cannula (fashioned from the needle of a 16G Medicut® Intravenous cannula). Two three-way taps provided ports for drug infusion and measurement of perfusion pressure. A two-channel infusion pump (Harvard Apparatus Co. Inc., Dover, Mass., USA) delivered drug through small-bore silicone tubing and short lengths of manometer tubing led to Model EM751 pressure transducers (Elcomatic, Glasgow, Scotland, UK). The transducers were linked to a hot-pen recorder (Devices Ltd., Welwyn Garden City, England, UK) which was calibrated by means of an aneroid sphygmomanometer connected to a pressure bottle.

FIGURE 2.1 *Diagrammatic representation of the hind-quarters perfusion apparatus.*



2.4.2 Perfusate

In these experiments, the perfusate was a modified Tyrode's buffer of the following ionic composition (mM) : Na^+ 148, K^+ 4.0, Ca^{2+} 1.84, Mg^{2+} 1.05, Cl^- 110, PO_4^{3-} 0.5, HCO_3^- 25 with glucose 11, pyruvate 1.8 and EDTA 0.027. The solution was gassed continuously with 95% O_2:5% CO_2 to produce a pH of 7.4±0.01 with the following gas tensions (mm Hg) : PO_2 571±8, PCO_2 36±0.7. The perfusate contained 3% high molecular weight Dextran (Mol. wt. approximately 90,000).

2.4.3 Surgical procedures

Rats were anaesthetised with intra-peritoneal sodium pentobarbitone (60 mg.kg body weight^{-1}). Both hind limbs were ligated firmly with a 2/0 silk suture placed just above the paw. The animal was taped in place on a heating pad and the abdominal cavity opened through a transverse incision. The abdominal aorta and inferior vena cava were

identified, gently dissected free of each other and a 4/0 silk suture placed loosely around the aorta proximal to the iliac bifurcation. Sodium heparin (250 IU.kg body weight^{-1}) was administered via the inferior vena cava or left renal vein. The proximal abdominal aorta was occluded with a bulldog clip and the flushed perfusion cannula quickly introduced into the aorta via an arteriotomy. The cannula was secured by tightening the loose 4/0 suture, the inferior vena cava was opened to permit free drainage and the animal was exsanguinated by severing the proximal abdominal aorta.

2.4.4 Protocol

Hind-quarters preparations were perfused in pairs. Perfusion was commenced at low flow (approximately 5 ml.100 g perfused tissue^{-1}.min^{-1}) to achieve maximal metabolic vasodilatation. The weight of perfused tissue was approximated by taking 40% of the total body weight. We confirmed this approximation to be reasonable in early experiments by comparing the dissected hind-quarters weight with body weight. After 10 minutes of low flow perfusion, the flow was gradually increased to reach a rate of approximately 10 ml.100 g perfused tissue^{-1}.min^{-1} at 20 minutes. This flow rate was maintained for the duration of the experiment. Mean perfusion pressure was recorded. Preparations with a baseline perfusion pressure exceeding 50 mmHg were rejected. During the dose-response study, drug infusion (maximum infusion rate 0.4 ml.min^{-1}, < 3% of the hind-quarters perfusion rate) was interrupted after each response plateau in order that perfusion pressure could be seen to decline satisfactorily to baseline levels before a further increment in dose was given. Plateaux were used to plot dose response curves. Preparations with a rising baseline, or where the baseline was elevated at the end of the protocol, were rejected.

2.4.5 Preliminary experiments

A number of preliminary experiments were performed to establish the range of concentrations of agonist and to examine the effects of selected antagonists. The

physiological neurotransmitter noradrenaline was used as the agonist in this and subsequent experimental models. Rauwolscine was used as an α_2-adrenoceptor antagonist and timolol as a non-selective β-adrenoceptor antagonist free of local anaesthetic effect with minimal intrinsic sympathomimetic activity. In Figure 2.2, the upper two dose-response relationships illustrate the effect of stimulation of the perfused rat hind-quarters with noradrenaline (0.5-10 μM) in the absence (n=10) and presence (n=10) of the two antagonists. In a third group of 6 rats, pre-perfusion with phenoxybenzamine 1 μM was seen to abolish the responses to noradrenaline in the presence of rauwolscine and timolol (Fig. 2.2, lowermost curve), thus indicating that these had been mediated by α_1-adrenoceptors. Body weights were similar in these three groups of laboratory chow fed animals (341±5, 340±6 and 349±4 g, all N.S.).

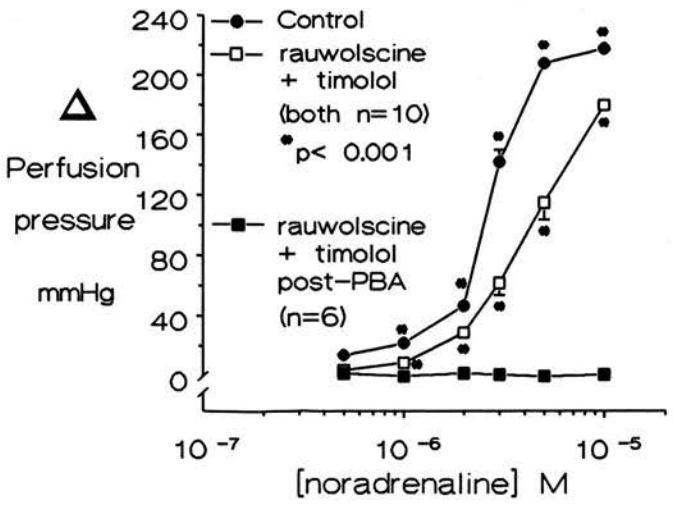


FIGURE 2.2 *The effect of rauwolscine (1 μM) and timolol (10 μM), and of these agents following phenoxybenzamine (PBA, 1 μM), on responses to noradrenaline (0.5-10 μM) in the perfused hind-quarters of the rat.*

In a small number of preparations vasoconstricted with noradrenaline to 60-75% of the maximum response, prompt relaxation (~50%) to acetylcholine 10 nM-1 mM was demonstrated, indicating endothelial integrity. This procedure was repeated

intermittently throughout the perfused hind-quarters experiments and remained satisfactory.

2.5 Isolated resistance artery studies

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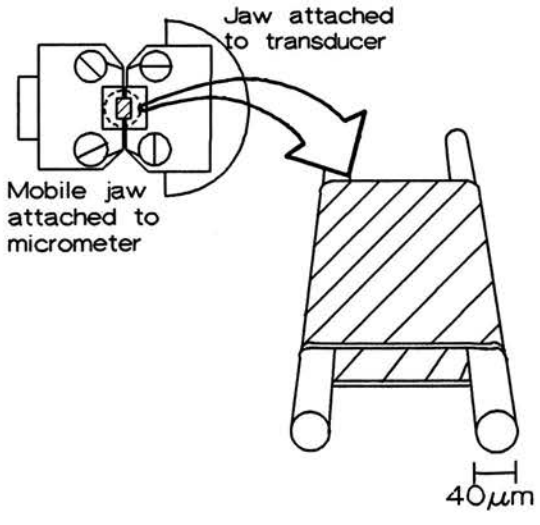
2.5.1 Experimental apparatus

The studies were carried out using an isometric myograph as described by Mulvany and Halpern [1977]. The apparatus contains a 10 ml organ chamber in which lie two pairs of jaws. The fixed medial jaws are attached to strain gauge transducers and the movable lateral jaws are adjusted with micrometer gauge handles. Each jaw has two screw heads which serve as anchoring points for a length of fine stainless steel wire (40 μm diameter). A pair is displayed diagrammatically in Figure 2.3.

FIGURE 2.3 *Diagrammatic representation of a vessel mounted in the isometric myograph.*



Removal of a perspex cover allows addition of buffer and drug to the organ chamber into which gas is bubbled continuously. The chamber is drained by suction and the apparatus maintained at the desired temperature by a water circulating system. The transducers were linked to a Grass Model 7D polygraph and were calibrated by measuring the pen deflection produced when a known mass on a small pivot was set to pull against a wire mounted on the transducer jaw.

2.5.2 Perfusate

The buffer used in these experiments was a physiological salt solution (PSS) of the following composition (mM) : NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, glucose 5 and EDTA 0.026. K-PSS (see 2.5.4) had the same composition but with an equimolar substitution of KCl for NaCl. Shortly before use, solutions were brought to 37° C in a water bath and were bubbled with 95% O_2:5% CO_2 to achieve a pH of 7.4.

2.5.3 Surgical procedures

Rats were sacrificed by stunning and cervical dislocation. Both hind limbs were rapidly removed by excision through the hip joint and care taken to preserve the inguinal area. The limbs were placed immediately in ice-cold buffer. One hind-limb at a time was pinned in a petri dish and repeatedly irrigated with ice-cold buffer whilst a section of a second order branch of the superficial femoral artery was dissected out with the aid of a binocular Nikon microscope. A 2-3 mm segment, free of side branches, was then separated from any accompanying vein and cleared of adventitia before being mounted as a ring in the myograph (Figure 2.3). The procedure was repeated to dissect and mount a second vessel.

2.5.4 Protocol

The vessels were allowed to equilibrate gradually to 37° C. Thereafter, resting tension - internal circumference relationships were determined in order to

calculate L_{100}, the internal circumference the vessel would maintain relaxed under a transmural pressure of 100 mmHg, using a Hewlett-Packard programmeable calculator. The effective transmural pressure was calculated after Laplace from:

$$P = T \div (L / 2\pi)$$

(P: pressure, kPa; T: tension, $mN.mm^{-1}$; L: internal circumference, mm)

The vessels were then set to normalised internal circumferences L_0, where $L_0 = 0.9\ L_{100}$, at which setting maximum contractile force can be achieved. Vessels were then activated by thrice stimulating with 10 μM noradrenaline in K-PSS. It has been shown that 10 μM noradrenaline induces maximal contractions in vessels of this calibre [Mulvany *et al.*, 1980]. Any vessel found to have an effective internal diameter (L_{100}/π) in excess of 300 μm, or unable to contract against an effective transmural pressure of 100 mmHg, was discarded. Following activation, dose-response relationships were determined. Vessels were exposed to increasing concentrations of agonist for three minutes at each concentration. The response plateau at the end of each three minute period was used to plot dose response curves. Where oscillatory changes were observed, the mean tension for the last 20 seconds was used. In the second part of the resistance artery studies, vessels were constricted to 60% of the maximum tension recorded during the preceding dose response study and relaxation brought about by acetylcholine was examined. Vessels demonstrating a contractile response to low doses of acetylcholine were considered to have been de-endothelialised by the mounting process and were accordingly rejected.

2.5.5 Preliminary experiments

To assess the appropriate dose range of agonist and to examine the effect of selected antagonists, a number of preliminary experiments were performed. As in the hind-quarters model, the agonist was noradrenaline. Timolol was used a non-selective β-adrenoceptor antagonist and yohimbine as an α_2-adrenoceptor antagonist. The

effect of stimulation of resistance arteries isolated from control rats with noradrenaline in the absence and presence of these antagonists is illustrated in Figure 2.4. Although the dose response relationships did not differ significantly, subsequent studies were nevertheless performed in the presence of α_2- and β-adrenoceptor antagonists. Relaxation of this preparation to acetylcholine and endothelial integrity are referred to above.

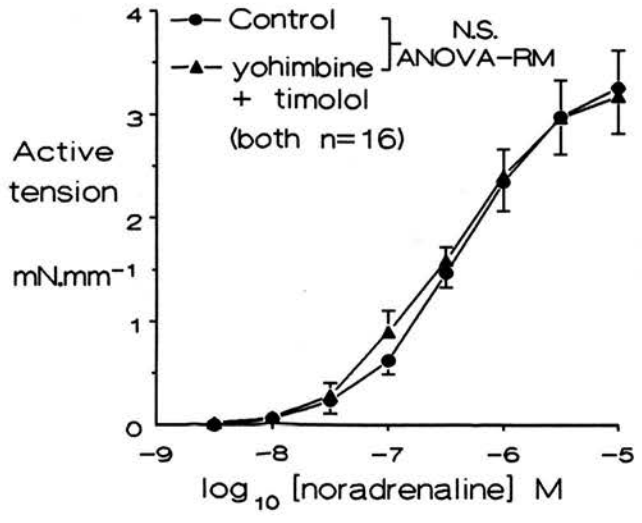


FIGURE 2.4 *Changes in active tension (mN.mm^{-1}) in response to noradrenaline (3 nM-10 μM), in the absence and presence of yohimbine (1 μM) and timolol (1 μM), of femoral resistance arteries isolated from the rat.*

The resistance artery experiments were performed in collaboration with Dr T.S. Lawal, Commonwealth Research Fellow, Department of Medicine, Leicester University, Leicester, England under the supervision of Dr A.M. Heagerty, Senior Lecturer in Medicine (now Professor of Medicine, University Hospital of South Manchester, Manchester, England).

2.6 Isolated heart perfusion studies

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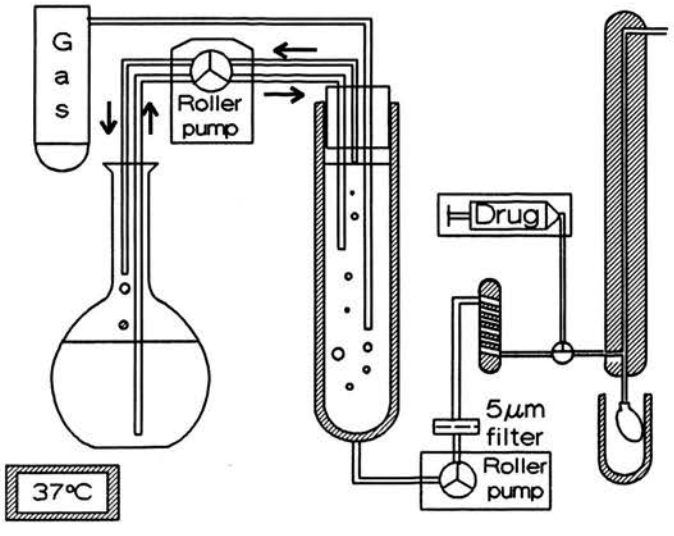
2.6.1 Perfusion apparatus

The method of perfusion of the isolated rat heart was modified from Langendorff [1895]. Apparatus was designed to reproduce the conditions described by Reibel *et al.* [1988] and is displayed diagrammatically in Figure 2.5. By means of a roller pump (Watson Marlow Ltd., Falmouth, Cornwall, England), buffer was circulated continuously between a 5 L flask and a 500 ml capacity gassing reservoir. A second roller pump drew oxygenated buffer of pH 7.4 from the reservoir and fed it in parallel through gas-impermeable tubing to a pair of glass heat exchangers. Buffer then passed via three-way taps, where drug could be infused, into 60 cm glass columns, open at the top and bearing a three-way tap and a grooved 3.5 mm metal cannula at the lower end.

FIGURE 2.5 *Diagrammatic representation of the isolated heart perfusion apparatus.*



Once cannulated, the hearts were surrounded by a small chamber with a drainage hole. The gassing reservoir, heat exchangers, columns and heart chambers were all water-jacketted and in series with a hot water circulator which maintained the perfusate at 37° C. The rate of circulation of buffer to and from the gassing reservoir exceeded the rate of withdrawal for perfusion. The perfusion rate was held constant and always exceeded the maximum coronary flow rate. This strategy permitted the use of drug solutions of fixed concentration, delivered through fine bore silicone tubing by a two-channel infusion pump (Harvard Apparatus Co. Inc., Dover, Mass., USA). LVP was transmitted via fine-gauge manometer tubing to Model EM751 pressure transducers from 1 mm bore grooved stainless steel cannulae inserted at the apices of the hearts. Calibration was carried out as described in section 2.4.1. The signals from the transducers were fed to a Mingograph 34B four-channel ECG recorder (Siemens Elema, Stockholm, Sweden) through a purpose-built pre-amplifier/signal differentiator (Department of Medical Physics and Engineering, University of Edinburgh).

2.6.2 Perfusate

A modified Krebs-Henseleit buffer of the following composition (mM) was used for the perfusion of the isolated heart: Na^+ 140, K^+ 5.5, Ca^{2+} 2.4, Mg^{2+} 1.0, Cl^- 120, PO_4^{3-} 0.4, HCO_3^- 25, glucose 5.0. The buffer was continually gassed with 95% O_2: 5% CO_2 to achieve a pH of 7.37±0.00 and gas tensions (mm Hg) of PO_2 576±5 and PCO_2 38.6±0.2.

2.6.3 Surgical procedures

Rats were anaesthetised with intra-peritoneal sodium pentobarbitone (60 $mg.kg^{-1}$). The abdominal cavity was opened transversely to expose the diaphragm and sodium heparin (250 $IU.kg^{-1}$) was administered via the inferior vena cava or left renal vein. In rapid sequence; the diaphragm was slit, the rib cage opened and the heart with the proximal great vessels still attached removed and placed in ice-cold buffer to effect

cardioplegia. As soon as beating ceased, the aortic root was drawn swiftly over the perfusion cannula and secured firmly with a 2/0 silk suture as perfusion commenced. The elapsed time between opening the diaphragm and the aortic perfusion was approximately 90 seconds. After gentle removal of pericardial fat and connective tissue and separation of any attached lung tissue from the pulmonary vessels, the cardiac apex was punctured with fine dissecting forceps and the 1 mm pressure cannula introduced to the left ventricle. Spent buffer dripping from the heart was taken as coronary venous effluent for the purposes of estimating coronary flow rates and lactate assay.

2.6.4 Protocol

Hearts were hung in pairs. In the dietary studies, the sequence of paired perfusions was such that the perfusion channel (left/right) was alternated for hearts from the same dietary group (e.g. control/n-3 PUFA ⇨ n-6 PUFA/control ⇨ n-3 PUFA/n-6 PUFA etc.). After stabilising for 15-20 minutes from the time of aortic cannulation, the coronary venous effluent was collected into 10 ml tubes of known weight. Baseline peak LVP, $LVdP.dt^{-1}$ and heart rate were recorded. Hearts were then exposed to increasing concentrations of agonist and cumulative dose response curves were constructed with pressure and heart rate data recorded three minutes after each dose increment. Following the final dose, pressures were observed for a decline to baseline values. This protocol was completed within 35-40 minutes. Hearts which developed ventricular fibrillation, sustained ventricular tachycardia or excessive bradycardia were rejected as were those which did not recover baseline pressure following the administration of agonist. If baseline lactate production was subsequently found to exceed 1 $\mu mol.min^{-1}.g$ wet heart $weight^{-1}$, hearts were also excluded from further analysis.

2.6.5 Preliminary experiments

A small number of preliminary experiments were performed to confirm the appropriate range of agonist and to examine the nature of the responses elicited. The hearts of 6 Sprague-Dawley rats were perfused with buffer containing timolol 10 μM and prazosin 1 μM. The infusion of noradrenaline 0.1-2.5 μM failed to elicit any increase in peak LVP (Fig. 2.6). A further 6 hearts were perfused and responses to noradrenaline 2.5 μM, the maximum dose used in subsequent experiments, in the presence of timolol 10 μM, were obtained. Increasing the concentration of timolol ten-fold to 0.1 mM had no effect on the increment in peak LVP in response to noradrenaline (Fig. 2.6, inset).

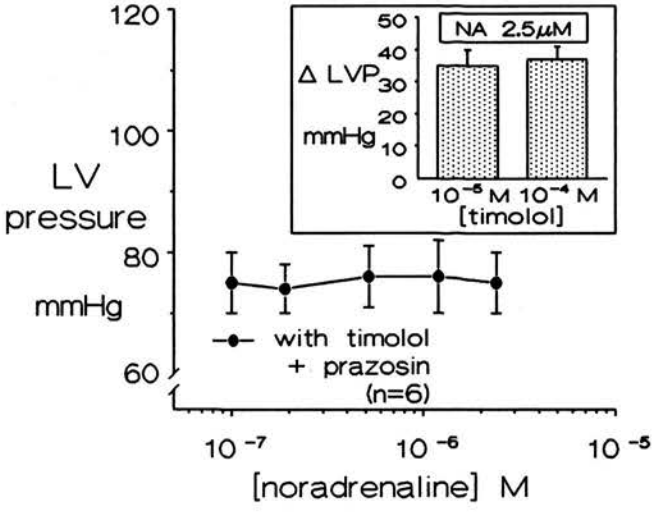


FIGURE 2.6 *Response of peak LVP to noradrenaline (0.1-2.5 μM), in the presence of timolol 10 μM and prazosin 1 μM and (inset) to noradrenaline 2.5 μM in the presence of timolol 10 μM and 0.1 mM, in hearts isolated from chow-fed rats.*

It was concluded from these preliminary studies that the responses of the isolated perfused heart to noradrenaline in the presence of timolol 10 μM were mediated by α_1-adrenoceptors

2.6.6 Chemical denervation protocol

One set of perfusions was performed with hearts isolated from chemically denervated rats fed experimental diets (Chapter 5). After six weeks of the eight week feeding period, 6-hydroxy-dopamine chloride was administered intraperitoneally according to the following regimen, adapted from Allely [1983].

Day 1..... *50 mg.kg^{-1}*

Day 2..... *50 mg.kg^{-1}*

Day 8..... *100 mg.kg^{-1}*

Day 9..... *100 mg.kg^{-1}*

The efficacy of this regimen has been carefully established by means of histochemistry. Allely [1983] used an autofluorescence microscopy/sucrose-phosphate-glyoxylic acid method [de la Torre and Surgeon, 1976] to demonstrate the absence of sympathetic neuronal vesicles in the hearts of treated animals.

The 6-hydroxy-dopamine was prepared as described in section 2.3. The perfusion studies took place 11 days after the last dose of 6-hydroxy-dopamine.

2.7 Biochemical Analyses

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2.7.1 Analysis of adipose fatty acid composition

Approximately 10 mg of adipose tissue was homogenised in 5 ml isopropanol/heptane (4:1, v/v) using an Elvjehem-Potter glass homogeniser. Phospholipids and free fatty acids were separated by back-extraction from heptane into 0.05% KOH (1:1 potassium hydroxide/heptane, v/v). The triglyceride-containing heptane layer was washed with 8 ml isopropanol/0.05% KOH (4:1, v/v, previously washed in heptane, 4:1:3, v/v/v) before being transferred to Quickfit tubes and evaporated to dryness on a Buchi-R rotary evaporator. Base-catalysed transmethylation [Christie, 1982] was

initiated by adding 1 ml toluene with 2 ml 0.5 M sodium methoxide and incubating at 50° C for 10 minutes. After washing with 11 ml glacial acetic acid/water/hexane (1/5/5, v/v/v), the organic phase was evaporated to dryness and the methylesters were redissolved in 40 ml chloroform with 0.01% butylated hydroxytoluene. The solutions were transferred to GLC vials and stored at -20° C prior to analysis.

2.7.2 Analysis of cardiac total and fractionated phospholipids

Hearts were freeze-dried for 48 hours on an Edwards-Modulyo freeze dryer before being crushed to a fine powder with a mortar and pestle. Approximately 150 mg of the powder from each heart was weighed out and placed in a Quickfit tube (20 x 125 mm). Phospholipids were extracted according to Folch [1957] using 21 ml chloroform/methanol (2:1, v/v) which was filtered into a second Quickfit tube (20 x 150 mm) before rinsing with a further 9 ml 2:1 chloroform/methanol. Then, 7 ml 0.88% KCl was added and the aqueous phase was removed. The organic phase was subsequently dried down under vacuum in a 1 L Buchi flask on the Buchi R rotary evaporator. The lipid extract was dissolved in 6 ml 2:1 chloroform/methanol, placed in a Quickfit tube, dried under vacuum once more and redissolved in 1 ml 2:1 chloroform/methanol using a Hamilton syringe.

Total phospholipid was separated into PC, PE, PI and PS by thin layer chromatography. Aliquots of extract (100 µl) were streaked across Whatman LK5 plates (20 x 20 cm, preadsorptive area) which had been pre-developed with chloroform/methanol (1:1, v/v), impregnated with 1% boric acid and activated at 100° C for 60 minutes. Plates were developed in chloroform/methanol/water/ammonia (120:75:6:2, v/v/v/v) in a paper-lined tank for 60 minutes and dried with a commercial hair-dryer. The plates were then sprayed with 0.1% dichlorofluoroscein to allow individual phospholipid bands to be identified under ultraviolet light (365 nm). The bands for the five phospholipid fractions were scraped off and placed in Quickfit tubes

before the addition of internal standard. For PC and PE fractions, 70 μg phosphatidylcholine C17:0 was added and for PS and PI, 2 μg was added. Transmethylation and extraction of methylesters was carried out as described in section 2.7.1.

2.7.3 Gas liquid chromatography

The system used was a Pye Unicam Series 204 gas chromatograph (Philips Analytical, Cambridge, England) fitted with a PU4700 autoinjector. A gas column (1.5 m, 2 mm internal diameter) packed with a stationary phase of 10% SP2330 on 100/120 mesh chromosorb WHW (Supelco) was used for all separations. GLC vials containing solutions of methyl esters were allowed to equilibrate to room temperature before being placed on the autoinjector. Samples of PS and PI (5 μl) were injected on to the column with a 10 μl syringe and samples of PC and PE (1 μl) with a 1 μl syringe. The system settings and temperature programme were as follows.

Injector temperature	220° C	
Detector temperature	300° C	
Column temperature	180° C	
Hydrogen gas flow	50 l.min^{-1}	
Carrier gas flow	50 l.min^{-1}	
Air flow	550 l.min^{-1}	
Programme	initial minutes	3
	rate (°C.min^{-1})	3
	final °C	250
	final minutes	5

Initially, the system was linked to a Trilab Model II integrator (Trivector) but early in the course of this project new chromatography software was introduced (Perkin Elmer Nelson Systems Inc., Cupertino, CA 95014, USA) run on an IBM PS/1 Model 30/286

computing system. Fatty acid methyl ester peaks had been identified originally using authentic standards and thereafter retention times with respect to the 17:O methyl esters were used. Chromatograms were inspected on screen prior to saving on disk and printing.

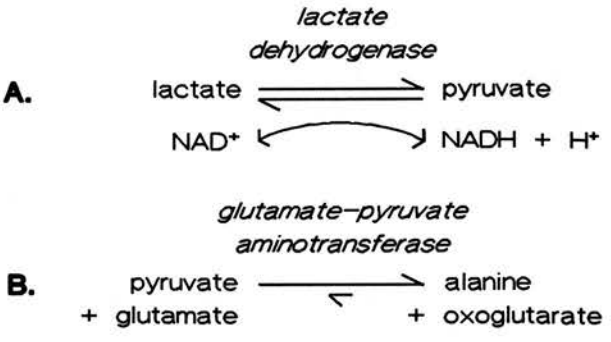
2.7.4 Fatty acid nomenclature, data and description of results

Fatty acids are described using a numbered abbreviation according to the following convention. The first number is the carbon chain length after which a dotted colon precedes the number of carbon:carbon double bonds. An n- designation indicates the number of carbon atoms from the methyl terminal to the first double bond. Thus, linoleic acid (*cis,cis,* 9,12-octadecadienoic acid) is abbreviated to 18:2, n-6 and timnodonic acid (more commonly known as *all cis,* 5,8,11,14,17-eicosapentaenoic acid) to 20:5, n-3.

Routinely, 19 individual fatty acid species, ranging from 14:0 to 22:6, n-3, were reported. Values for 14 of these were used to calculate double bond indices, and for 10 to calculate n-6:n-3 fatty acid ratios, with respect to the experimental diets. For clarity, the results which are later described are restricted to the double bond indices, the n-6:n-3 ratios and data for four fatty acid species of particular interest. These are the following. First, linoleic acid (18:2, n-6), as this is the principal n-6 fatty acid offered by the n-6 PUFA enriched diets. Second, arachidonic acid (20:4, n-6), as this elongation/desaturation product of 18:2, n-6 has considerable biological importance as the substrate for prostanoid synthesis. Third, eicosapentaenoic acid (20:5, n-3), as this species is not present in plant oils but was provided by the diets containing fish oil. Fourth, docosahexaenoic acid (22:6, n-3), as this was also provided by the fish oil diets and is an important n-3 fatty acid in cardiac total and fractionated phospholipids.

2.7.5 Measurement of coronary venous lactate

Coronary venous lactate concentrations were measured using a commercial kit (Boehringer) on a Cobas Bio centrifugal analyser (Roche Diagnostica, F. Hoffman-La Roche and Co., Basle, Switzerland). In this assay, lactate concentration is directly proportional to NADH (measured spectrophotometrically: 340 nm at 37° C) produced during the conversion of lactate to pyruvate in the presence of excess NAD^+, as shown; reaction **B.** is effectively unidirectional and reaction **A.** is pulled to completion. As the assay kit was designed for clinical use, where lactate levels can be considerably higher, it was possible to use NAD^+ at 40% of the recommended concentration.



A 1 mM lactate solution was assayed in triplicate in each run as an internal standard and quality control was provided by the repeated assay of Precinorm-S (Boehringer, mean concentration 880 μM). The interassay coefficient of variation was 10%.

2.7.6 Measurement of cardiac noradrenaline content

The noradrenaline content of cardiac tissue was measured by a radioenzymatic method modified from Da Prada and Zurcher [1976]. Hearts for analysis were freeze-dried and crushed as described in section 2.7.2. Approximately 10 mg of the powdered tissue was weighed into a Beckman tube to which 1 ml 0.3 M $HClO_4$ was added. The sample was whirlimixed before centrifuging at 15,000 rpm for 5 minutes. The supernatant was removed and stored at -40° C pending assay. Noradrenaline in the samples was converted to the 3-O-methylated derivative normetanephrine using rat liver catechol-O-methyl transferase in the presence of the tritiated methyl donor

^{3}H-methyl-S-adenosyl methionine. The reaction product, ^{3}H-normetanephrine, was purified by selective ion pair extraction with tetraphenylborate, separated by thin layer chromatography and subsequently oxidised to ^{3}H-vanillin which was counted in a liquid scintillation counter. The coefficient of variation of the assay was 7%.

2.7.7 Assay of coronary venous 6-*oxo*-PGF$_{1\alpha}$

Coronary venous 6-*oxo*-PGF$_{1\alpha}$ concentrations were measured by radioimmunoassay. On stabilisation of the perfused hearts, timed samples of coronary venous effluent were collected into 10 ml tubes of known weight which were weighed immediately and frozen by placing in dry ice. Pending analysis, tubes were stored at -40° C.

A standard curve was constructed by using known concentrations of 6-*oxo*-PGF$_{1\alpha}$ (0.01-5.12 ng.ml^{-1}) in diluent composed of 0.05 M *tris* buffer (pH 6.8) containing gelatin (1 g.l^{-1}). Aliquots of 500 µl were placed in tubes in triplicate. From the coronary venous samples, 100 µl and 200 µl aliquots were taken, placed in tubes in duplicate and made up to 500 µl with diluent. Tritiated 6-*oxo*-PGF$_{1\alpha}$ (50 µl, specific activity 0.125 µCi.ml^{-1}) was added to the standard curve tubes and coronary venous sample tubes, to eight tubes containing diluent alone; four for zero standards and four for counting standards, and to four tubes containing 10 ng.ml^{-1} 6-*oxo*-PGF$_{1\alpha}$ to assess non-specific binding of the tracer. An empty tube was used for machine background. Rabbit antibody to 6-*oxo*-PGF$_{1\alpha}$ (50 µl) was then added to all but the counting standard and machine background tubes and the tubes were whirlimixed before incubating at room temperature for 1 hour. Normal rabbit serum (50 µl, 1:100) and donkey anti-rabbit serum (50 µl, 1:10) were added to the tubes containing antibody and all tubes were incubated overnight at 4° C. Thereafter, tubes containing antibody + serum were centrifuged at 2,500 rpm, 4° C, for 30 minutes and the supernatant carefully discarded. Scintillant (2.5 ml), composed of 10.5 g 2,5-diphenyloxazole + 900 ml 2-ethoxyethanol + 1.5 l toluene, was added to all tubes.

After whirlimixing, the tubes were placed in a scintillation counter and counted for 4 minutes each, commencing with machine background and counting standards tubes. The percentage of tracer bound was calculated according to:

(counts in tube ÷ average standard counts) x 100

The standard curve was plotted and the concentrations of 6-*oxo*-$PGF_{1\alpha}$ in the coronary venous samples were obtained by extrapolation.

The cross reactivity of the 6-*oxo*-$PGF_{1\alpha}$ antibody was 4.2% for PGE_2, 1.1% for PGE_1 and 0.43% for $PGF_{1\alpha}$. For other prostanoids and thromboxane, the cross reactivity was < 0.1%.

This assay was performed courtesy of Dr N. Poyser, Senior Lecturer, Department of Pharmacology, University of Edinburgh.

2.8 Statistical methods

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Statistical analyses were carried out using commercial software (Release 7, Minitab Inc., State College, PA16801, USA) run on an IBM PS/2 Model 55SX computing system. All data are expressed as mean ± standard error of the mean (s.e.m.).

Continuous variables, for example animal weight, coronary flow and lactate measurement, were normally distributed and were compared with a two sample *t* test. The effect of the experimental diets on adipose tissue and tissue phospholipid fatty acid composition was screened by one-way analysis of variance using a digital code (levels) for diet. Where significant variation was apparent, a two sample *t* test corrected for multiple comparisons was applied for individual fatty acid species, double bond indices and n-6:n-3 ratios. A Mann-Whitney test was used to corroborate significant differences in double bond indices and n-6:n-3 ratios, as these derived parameters might not be normally distributed.

Several means of analysing the dose-response data, with or without transformation, were tested and found to be unsatisfactory. Hand drawn curves might not be consistent, single and double reciprocal plots fitted the data poorly and a more complex unconstrained four-parameter logistic regression model fitted some experimental data well but other equally sound data not at all. Consequently, dose-response data, displayed as absolute response *versus* $\log_{10}$ concentration of agonist, were simply interpolated and the relationships so defined were interrogated by analysis of variance for repeated measures using a three factor design which included diet, dose and (random) rat effects. This analysis also indicated whether significant variation between dose-response relationships was associated with non-parallelism, reflecting an effect of diet on the "rate" at which maxima were achieved. Where significant variation was evident, the points corresponding to specific concentrations of agonist were subjected to a two sample *t* test corrected for multiple comparisons.

A similar analysis of variance for repeated measures, taking account of the random effects of time and potential changes in the rat population, was used to assess whether fractionated phospholipid fatty acid composition related to a given experimental diet varied between experiments.

It was clearly important to attempt to correlate any changes in pharmacological response with respect to diet with the changes in fatty acid composition brought about by the diet. A best subsets regression analysis was used to canvas for possible relationships between the four fatty acid species of particular interest, the double bond indices and the n-6:n-3 ratios, singly and in multiple combination, and the tissue responses.

Throughout, significance was accepted at the 5% level.

2.9 Materials

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All materials were of at least analytical grade.

Aldrich Chemical Company, Gillingham, Dorset, England
CH_3ONa

BDH (Chemicals) Ltd., Poole, Dorset, England
$CaCO_3$, $FeC_6H_5O_7$, KIO_3, NaCl, $NaHCO_3$, $ZnCl_2$, perchloric acid.

Boehringer Corporation Ltd., Lewes, E. Sussex, England
Enzymatic kit for lactate

Leo Laboratories Ltd., Aylesbury, England.
Sodium heparin

May and Baker Ltd., Dagenham, England
Sodium pentobarbitone

Merck, Burnfield Avenue, Thornliebank, Glasgow, Scotland
$CHCl_3$, CH_3OH, glacial acetic acid, heptane, isopropanol, KOH, NH_3, toluene

Carl Roth, Karlsruhe, Germany
Rauwolscine hydrochloride

Sigma Chemical Co., Poole, Dorset, England
Butylated hydroxytoluene, $CaCl_2.2H_2O$, $CuCl_2.2H_2O$, dichlorofluorescein, KCl, $KHCO_3$, KH_2PO_4, $MgCl_2.6H_2O$, $MgHPO_4.3H_2O$, $MnSO_4$, $Na_3C_6H_5O_7.2H_2O$, $NaH_2PO_4.H_2O$, C17:0-phosphatidylcholine, ^{3}H-methyl-S-adenosylmethionine.
Acetylcholine HCl, dextran (mol. wt. 60k-90k), 6-hydroxydopamine HCl, isoprenaline HCl, noradrenaline bitartrate, noradrenaline HCl, prazosin HCl, timolol maleate, yohimbine HCl

Smith Kline Beecham, Welwyn Garden City, Hertfordshire, England
Phenoxybenzamine HCl

Chapter 3

Dietary polyunsaturated fatty acids and peripheral vasoconstriction

3.1 Introduction

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In man, vascular responses to noradrenaline and to cold provocation in Raynaud's disease can be attenuated by n-3 PUFA [Lorenz *et al.*,1983, di Giacomo *et al.*, 1989] and diets with a high content of 20:4, n-6 reduced forearm vasoconstrictor responses to a cold pressor test [Butcher *et al.*, 1990]. Animal studies of vascular reactivity have focused on the effects of n-3 PUFA and there are inconsistencies. Vascular responses to catecholamines and to angiotensin II have been reduced, not altered and even increased by n-3 PUFA [Lockette *et al.*, 1982, Yin *et al.*, 1988, Yin *et al.*, 1991, Yoshimura *et al.*, 1986, Kenny *et al.*, 1990].

Interpretation of conflicting animal results is made difficult by differences in experimental diets. The amount of n-3 PUFA administered in these studies varied widely and doses equivalent to at least 40 $g.day^{-1}$ in the average man were given, more than five times the daily n-3 PUFA intake of Greenland Eskimos [Bang and Dyerberg, 1985]. This degree of n-3 PUFA supplementation is poorly tolerated by man and is clearly not realistic. Control diets are often laboratory chow which is of low fat content, 5% to 10% of dietary calories, and disproportionately rich in 18:2, n-6, thus not relevant to man. Commonly, the effects of different fatty acids are assessed by simply adding the source fat or oil to laboratory chow, but this practice is not without risk. The use of highly saturated fats may precipitate essential fatty acid

deficiency, which is associated with raised BP [Cox *et al.*, 1982]. Also, addition of fats or oils to laboratory chow compromises mineral and vitamin content. Dietary intake of Na^+ and K^+, for example, is likely to be reduced. These problems can be avoided by the use of semi-synthetic diets with an adequate 18:2, n-6 content (approximately 3% of dietary calories).

Our intention was to investigate the effects of PUFA in diets representative of what is, or might be, consumed by man on vasoconstriction in the peripheral vascular bed. In the first of two studies, we examined the effect of a moderate dietary supplement of n-3 PUFA (+ 0.4% dietary calories, equivalent to 3-4 g daily in the average man) on the responses to α_1-adrenoceptor stimulation in the rat hind quarters perfused at constant flow. In the second study, we examined the effect of increasing the dietary proportion of n-6 PUFA (to a P/S ratio of 2.0) in the same experimental model. For the control in these two experiments, we used a semi-synthetic diet deriving 40% of dietary calories from fat (P/S ratio 0.3), typical of the fat intake of middle-aged Scottish men [Thomson, 1984].

3.2 Methods

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3.2.1 Dietary n-3 PUFA

Thirty-six Lew rats were assigned at random to two groups, both of which were fed the P/S 0.3 diet. Half of the animals received a daily oral dose of fish oil and half received a control oral dose of olive oil. As discussed in section 2.2.4, the olive oil was not supplementary to the diet. The feeding period was eight weeks. The composition of the two final diets is shown in Table 3.1. At the end of the feeding period, the surgical procedures and protocol of sections 2.4.3 and 2.4.4 were followed. Control and n-3 PUFA rats were paired for the perfusions and the respective perfusion channel, right or left, was alternated run to run. Increments in perfusion pressure in response to noradrenaline 2-10 μM in the presence of timolol 10 μM and rauwolscine 1 μM were recorded. There was one preparation failure (n-3 PUFA group) and one

preparation was excluded from further analysis on the basis of a rising baseline pressure (control group).

TABLE 3.1 *Calculated fat composition (%) of the control and n-3 PUFA experimental diets.*

	Diet	
Fatty acid classes	Control	n-3 PUFA
Saturates	20%	20%
Monoenes	14%	14%
n-6 PUFA	6%	6%
n-3 PUFA	-	0.4%
Total	40%	40.4%

Values represent the proportion of dietary calories supplied by different classes of fatty acid.

3.2.2 Dietary n-6 PUFA

In a separate study, 24 Sprague-Dawley rats were randomly assigned to two groups and fed either the control diet or a diet of P/S ratio 2.0. These animals were not orally dosed. The composition of the diets is shown in Table 3.2.

TABLE 3.2 *Calculated fat composition (%) of the control and n-6 PUFA experimental diets.*

	Diet	
Fatty acid classes	Control	n-6 PUFA
Saturates	20%	8%
Monoenes	14%	14%
n-6 PUFA	6%	18%
n-3 PUFA	-	-
Total	40%	40%

Values represent the proportion of dietary calories supplied by different classes of fatty acid.

At the end of the feeding period, the surgical procedures and protocol of sections 2.4.3 and 2.4.4 were followed. The perfusions were performed as described for the n-3 PUFA experiment.

The n-3 PUFA experiments were performed in association with Dr Carol Sargent of the Cardiovascular Research Unit who was investigating the effect of diet on ventricular arrhythmia in the isolated perfused rat heart [Sargent, 1990].

3.3 Results

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3.3.1 Dietary n-3 PUFA and perfusion pressure

Noradrenaline, in the presence of rauwolscine and timolol, brought about a substantial rise in perfusion pressure (Fig. 3.1). Increments in pressure were significantly attenuated in relation to the n-3 PUFA supplemented diet compared to the control diet ($p< 0.05$, ANOVA-RM). At any specific concentration of noradrenaline, there was no significant difference in response in relation to diet.

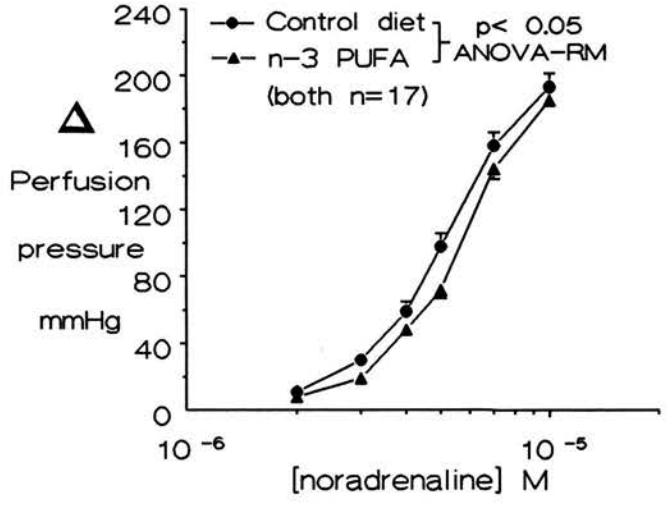

FIGURE 3.1 *Increments in perfusion pressure (mm Hg) in response to noradrenaline (2-10 μM), in the presence of rauwolscine (1 μM) and timolol (10 μM), in the perfused hind-quarters of rats fed experimental diets: n-3 PUFA.*

Baseline perfusion pressure was not altered by n-3 PUFA supplementation (n-3 PUFA: 42±1 mm Hg *versus* control: 41±1 mm Hg).

3.3.2 Dietary n-6 PUFA and perfusion pressure

In contrast to the findings of the n-3 PUFA experiment, increasing the n-6 PUFA content of the diet to achieve a P/S ratio of 2.0 had no significant effect on the rise in perfusion pressure in response to noradrenaline in the presence of rauwolscine and timolol (Fig. 3.2).

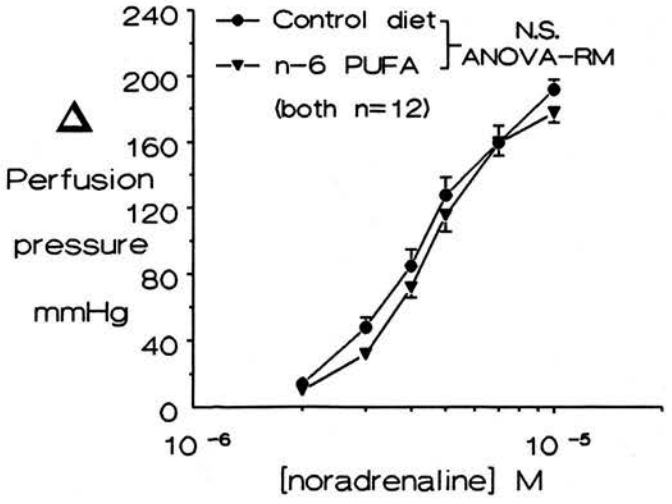

FIGURE 3.2 *Increments in perfusion pressure (mm Hg) in response to noradrenaline (2-10 μM), in the presence of rauwolscine (1 μM) and timolol (10 μM), in the perfused hind-quarters of rats fed experimental diets: n-6 PUFA.*

Baseline perfusion pressure prior to stimulation was unaffected by diet (n-6 PUFA: 41±2 mm Hg *versus* control: 44±1 mm Hg, NS).

3.3.3 Dietary n-3 PUFA: body weights and estimated hind-quarters flow rates

Final body weights and estimated hind-quarters perfusion flow rates (see section 2.4.4) were in close agreement for the n-3 PUFA supplemented and control diets (Table 3.3).

TABLE 3.3 *Final body weights (g) and estimated hind-quarters flow rates (ml.min^{-1}.100g tissue weight^{-1}) for rats fed experimental diets: n-3 PUFA.*

Diet	Body weight	Flow
Control	387±5	10.4±0.1
n-3 PUFA	386±7	10.4±0.2

Values: mean ± s.e.m., n=17 per group. There were no significant differences.

3.3.4 Dietary n-6 PUFA: body weights and estimated hind-quarters flow rates

Although there was a tendency toward greater body weights in the n-6 PUFA group, this was not significant (Table 3.4). Body weights were more widely distributed in this experiment but this did not lead to any significant difference in estimated hind-quarters perfusion flow rates.

TABLE 3.4 *Final body weights (g) and estimated hind-quarters flow rates (ml.min^{-1}.100g body weight^{-1}) for rats fed experimental diets: n-6 PUFA.*

Diet	Body weight	Flow
Control	379±16	10.7±0.4
n-6 PUFA	413± 9	9.7±0.2

Values: mean ± s.e.m., n=12 per group. There were no significant differences.

3.3.5 Dietary n-3 PUFA and the fatty acid composition of adipose tissue

Supplementation of the diet with n-3 PUFA did not affect the principal fatty acid 18:2, n-6 in adipose tissue, or the very small proportion of 20:4, n-6 (Table 3.5). The n-3 fatty acid species were not detected (< 0.1%).

TABLE 3.5 *Fatty acid composition of adipose tissue from rats fed experimental diets : n-3 PUFA.*

	Diet	
Fatty acid species	Control	n-3 PUFA
18:2, n-6	12.2±0.4	10.8±0.5
20:4, n-6	0.1±0.0	0.1±0.0
20:5, n-3	-	-
22:6, n-3	-	-

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. There were no significant differences.

3.3.6 Dietary n-6 PUFA and the fatty acid composition of adipose tissue

The n-6 PUFA diet brought about significant increases in the content of 18:2, n-6 and in the small proportion of 20:4, n-6 (Table 3.6). The n-3 species were not detected (< 0.1%).

TABLE 3.6 *Fatty acid composition of adipose tissue from rats fed experimental diets : n-6 PUFA.*

	Diet	
Fatty acid species	Control	n-6 PUFA
18:2, n-6	15.1±0.9[a]	30.9±0.4[a]
20:4, n-6	0.2±0.0[b]	0.4±0.1[b]
20:5, n-3	-	-
22:6, n-3	-	-

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. Superscript characters denote significant differences ($p < 0.01$) between individual fatty acid species.

3.3.7 Dietary n-3 PUFA and the fatty acid composition of cardiac total and fractionated phospholipid

Supplementation of the diet with n-3 PUFA did not alter the cardiac tissue content of total phospholipid or of separate phospholipid fractions compared to the control diet (Table 3.7). In total phospholipid, the proportion of 18:2, n-6 was not affected by n-3 PUFA supplementation but 20:4, n-6 was significantly decreased (Table 3.8). Levels of the n-3 species were significantly increased although 20:5, n-3 was barely detectable. The n-6:n-3 ratio was halved and the double bond index increased significantly.

TABLE 3.7 *Total phospholipid and phospholipid fractions in the hearts of rats fed experimental diets: n-3 PUFA.*

	Diets	
Phospholipid fractions	n-3 PUFA	Control
PC	5989±303	6737±605
PE	4297±209	4707±461
PI	567±48	641±90
PS	360±43	417±49
Total	13524±751	14070±1212

Values (mean ± s.e.m.) are expressed as μg fatty acids.g wet heart weight^{-1}, n=8 per dietary group. There were no significant differences.

TABLE 3.8 *Fatty acid composition, including the n-6:n-3 ratio and double bond index (DBI), of total phospholipid in the hearts of rats fed experimental diets: n-3 PUFA.*

Fatty acid species,	Diet	
n-6:n-3 ratio and DBI	Control	n-3 PUFA
18:2, n-6	18.3±0.7	17.7±0.7
20:4, n-6	27.6±2.2^{a}	22.3±1.1^{a}
20:5, n-3	0.1±0.0^{b}	0.2±0.0^{b}
22:6, n-3	10.6±0.5^{c}	13.3±0.5^{c}
n-6:n-3 ratio	3.9±0.5^{d}	2.0±0.2^{d}
DBI	225±3^{e}	234±2^{e}

Values: mean ± s.e.m., n=8 per dietary group. Data for fatty acids represent the percentage weight of total fatty acid species. Superscript characters denote significant differences ($p < 0.001$) between individual fatty acid species and parameters in relation to diet.

The fatty acid composition of fractionated phospholipid is shown in Table 3.9. In none of the fractions was 18:2, n-6 influenced by dietary n-3 PUFA supplementation. In PC,

PE and PS, but not in PI, the proportion of 20:4, n-6 was significantly decreased in relation to n-3 PUFA. Only in PC and PE was 20:5, n-3 detected where it was increased by supplementation which also significantly increased the relative amounts of 22:6, n-3 in all fractions.

TABLE 3.9 *Fatty acid composition of phospholipid fractions in the hearts of rats fed experimental diets: n-3 PUFA.*

Phospholipid fraction and fatty acid species		Diet: Control	Diet: n-3 PUFA
PC	18:2, n-6	8.6±0.7	8.9±0.8
	20:4, n-6	28.3±0.7[a]	24.7±0.6[a]
	20:5, n-3	0.1±0.0[b]	0.2±0.2[b]
	22:6, n-3	3.6±0.4[c]	5.9±0.5[c]
PE	18:2,n-6	4.7±0.4	4.7±0.3
	20:4, n-6	27.5±2.8[d]	22.6±1.0[d]
	20:5, n-3	-	0.3±0.2
	22:6, n-3	19.1±0.4[e]	31.2±0.7[e]
PI	18:2, n-6	4.1±0.3	4.0±0.5
	20:4, n-6	35.1±0.5	34.7±0.6
	20:5, n-3	-	-
	22:6, n-3	2.5±0.4[f]	4.2±0.5[f]
PS	18:2, n-6	2.1±0.3	2.3±0.5
	20:4, n-6	8.2±0.5[g]	6.6±0.7[g]
	20:5, n-3	-	-
	22:6, n-3	13.4±0.8[h]	19.1±1.0[h]

Values (mean ± s.e.m.) represent the percentage of total fatty acid species within each phospholipid fraction, n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between individual fatty acid species within phospholipid fractions in relation to diet.

In association with the above changes, the n-6:n-3 ratios fell, most strikingly so in PE where the decrease was threefold (Table 3.10). Double bond indices rose significantly in PC and PE with n-3 PUFA supplementation, but not in PI or PS.

TABLE 3.10 *Phospholipid fraction n-6:n-3 ratios and double bond indices (DBI) in the hearts of rats fed experimental diets: n-3 PUFA.*

Phospholipid fraction		Diet	
	n-6:n-3 ratios and DBI	Control	n-3 PUFA
	n-6:n-3 ratio	7.6±0.4[a]	3.0±0.5[a]
	DBI	190±1[b]	198±2[b]
PE	n-6:n-3 ratio	1.8±0.1[c]	0.6±0.2[c]
	DBI	278±2[d]	290±2[d]
PI	n-6:n-3 ratio	21.6±0.9[e]	8.4±0.7[e]
	DBI	187±4	188±2
PS	n-6:n-3 ratio	1.3±0.1[f]	0.6±0.1[f]
	DBI	190±3	196±3

Values: mean ± s.e.m., n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between parameters for individual phospholipid fractions in relation to diet.

3.3.8 Dietary n-6 PUFA and the fatty acid composition of cardiac total and fractionated phospholipid

Increasing the dietary content of n-6 PUFA did not alter the cardiac tissue concentrations of total phospholipid or of the separate phospholipid fractions (Table 3.11). The proportion of 18:2, n-6 was significantly increased in total phospholipid in relation to the n-6 PUFA rich diet whereas that of 20:4, n-6 was not (Table 3.12). There was a small but significant decrease in 22:6, n-3 and 20:5, n-3 was not detected. A 50% increase was apparent in the n-6:n-3 ratio and the double bond index fell significantly.

TABLE 3.11 *Total phospholipid and phospholipid fractions in the hearts of rats fed experimental diets: n-6 PUFA.*

	Diets	
Phospholipid fractions	Control	n-6 PUFA
PC	4576±481	4940±546
PE	3455±398	3760±432
PI	496±41	539±35
PS	236±52	240±61
Total	10054±871	10983±943

Values (mean ± s.e.m.) are expressed as μg fatty acids.g wet heart weight^{-1}, n=8 per dietary group. There were no significant differences.

TABLE 3.12 *Fatty acid composition, including the n-6:n-3 ratio and double bond index (DBI), of total phospholipid in the hearts of rats fed experimental diets: n-6 PUFA.*

Fatty acid species,	Diet	
n-6:n-3 ratio and DBI	Control	n-6 PUFA
18:2, n-6	16.4±0.4^{a}	21.3±0.5^{a}
20:4, n-6	25.2±0.3	24.4±0.4
20:5, n-3	-	-
22:6, n-3	9.6±0.3^{b}	8.7±0.2^{b}
n-6:n-3 ratio	4.1±0.7^{c}	6.4±0.3^{c}
DBI	228±1^{d}	222±2^{d}

Values: mean ± s.e.m., n=8 per dietary group. Data for fatty acids represent the percentage weight of total fatty acid species. Superscript characters denote significant differences between individual fatty acid species and parameters in relation to diet ($0.001<p< 0.05$).

The fatty acid composition of cardiac fractionated phospholipid with respect to dietary n-6 PUFA is shown in Table 3.13. The proportion of 18:2, n-6 rose significantly in all

fractions. However, not only was 20:4, n-6 unaffected in PC, PI and PS but it fell in PE. A 20% decrease was seen in 22:6, n-3 in all fractions and 20:5, n-3 was not detected (< 0.1%).

TABLE 3.13 *Fatty acid composition of phospholipid fractions in the hearts of rats fed experimental diets: n-6 PUFA.*

Phospholipid fraction and fatty acid species		Diet	
		Control	n-6 PUFA
PC	18:2, n-6	6.5±0.6[a]	8.0±0.5[a]
	20:4, n-6	29.8±0.7	30.2±0.6
	20:5, n-3	-	-
	22:6, n-3	3.9±0.3[b]	3.0±0.4[b]
PE	18:2,n-6	4.5±0.4[c]	6.7±0.5[c]
	20:4, n-6	25.7±0.6[d]	23.1±0.3[d]
	20:5, n-3	-	-
	22:6, n-3	19.6±0.5	15.2±0.7
PI	18:2, n-6	4.2±0.2[e]	6.9±0.2[e]
	20:4, n-6	35.1±0.4	35.8±0.3
	20:5, n-3	-	-
	22:6, n-3	1.3±0.3[f]	1.0±0.4[f]
PS	18:2, n-6	2.2±0.3[g]	3.6±0.5[g]
	20:4, n-6	7.7±0.4	7.5±0.8
	20:5, n-3	-	-
	22:6, n-3	14.1±0.7	12.1±0.3

Values (mean ± s.e.m.) represent the percentage of total fatty acid species within each phospholipid fraction, n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between individual fatty acid species within phospholipid fractions in relation to diet.

The n-6:n-3 ratios in fractionated phospholipid increased by 50%, as in total phospholipid, with dietary n-6 PUFA and the double bond indices fell, though not significantly (Table 3.14).

TABLE 3.14 *Phospholipid fraction n-6:n-3 ratios and double bond indices (DBI) in the hearts of rats fed experimental diets: n-6 PUFA.*

Phospholipid fraction		Diet	
	n-6:n-3 ratios and DBI	Control	n-6 PUFA
PC	n-6:n-3 ratio	7.7±0.5[a]	11.9±0.6[a]
	DBI	188±2	185±3
PE	n-6:n-3 ratio	1.7±0.1[b]	2.3±0.1[b]
	DBI	274±3	270±4
PI	n-6:n-3 ratio	20.5±0.8[c]	31.3±1.1[c]
	DBI	189±4	187±3
PS	n-6:n-3 ratio	1.4±0.1[d]	2.1±0.2[d]
	DBI	194±5	190±4

Values: mean ± s.e.m., n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between parameters for individual phospholipid fractions in relation to diet.

3.4 Discussion

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In the first of these two studies, supplementation of a 40% fat calorie diet of P/S ratio 0.3 with n-3 PUFA was found to attenuate responses to noradrenaline in the hind-quarters of Lew rats perfused at constant flow. The responses to noradrenaline, recorded as increases in hind-quarters perfusion pressure, were obtained in the presence of rauwolscine and timolol and were previously shown to be abolished by phenoxybenzamine. Thus, we infer that these responses were mediated by α_1-adrenoceptors. The fatty acid composition of the perfused arterial bed could not be determined due to the volume of tissue required, but analysis of cardiac fractionated

phospholipid fatty acid composition revealed numerous statistically significant changes in relation to dietary n-3 PUFA.

In the second study, an n-6 PUFA diet, P/S ratio 2.0, was not associated with any difference in response to α_1-adrenoceptor stimulation in the perfused hind-quarters of Sprague Dawley rats. An increase in adipose tissue 18:2, n-6 confirmed the consumption of the P/S 2.0 diet which also brought about significant changes in cardiac fractionated phospholipid fatty acid composition.

The results with respect to n-3 PUFA are in agreement with the findings of previous studies in rat vessels [Lockette *et al.*, 1982, Yin *et al.*, 1991, Malis *et al.*, 1991] where considerably higher doses of n-3 PUFA (10- to 15-fold) were used. However, neither our study nor these others reveals the mechanism by which supplementation of the diet with n-3 PUFA brings about attenuation of vascular responses to α_1-adrenoceptor stimulation. Curiously, despite much speculation regarding potential mechanisms in these published reports, only Lockette *et al.* [1982] present data on the changes in tissue fatty acid composition following their highly n-3 PUFA enriched experimental diet. These data are limited to total lipid analysis of aortic tissue where 5- to 7-fold increases in n-3 PUFA species were observed. We documented numerous changes of lesser degree in cardiac fractionated phospholipid fatty acid composition but the extrapolation of our cardiac data to vascular tissue must be cautious. Few studies have compared the effects of dietary PUFA on cardiac and vascular tissue but it has been shown that following long term feeding of a fish oil diet to marmosets, cardiac and aortic total lipid fatty acid composition differed only with respect to 22:6, n-3 which was relatively higher in cardiac tissue [Charnock *et al.*, 1992]. In the same study, enrichment of the diet with sunflower seed oil (a source of n-6 PUFA) was associated with a greater proportion of 18:2, n-6 in cardiac than aortic tissue but other fatty acid species were similar.

If the changes in cardiac tissue phospholipid fatty acid composition we describe in relation to n-3 PUFA supplementation also occurred in the peripheral arterial bed, vasoconstriction following α_1-adrenoceptor stimulation could be affected directly or

indirectly. The incorporation of n-3 PUFA in the PC and PE fractions, which together represent approximately 70% of cell membrane phospholipid, was associated with significantly greater unsaturation and reduced levels of 20:4, n-6. Unsaturation may render the cell membrane more fluid, affecting events such as agonist-receptor coupling, for example. Reductions in 20:4, n-6 may compromise the availability of substrate for the synthesis of vasoconstrictor prostanoids. Interestingly, in the PI fraction, linked with α_1-adrenoceptor signal transduction, although 22:6, n-3 was increased and the n-6:n-3 ratio decreased, the proportion of 20:4, n-6 did not fall with dietary n-3 PUFA, as it is commonly assumed to do on the basis of total phospholipid data, and the double bond index did not rise. Our data indicate that the PI fraction is relatively resistant to dietary influence.

Despite the large increase in dietary n-6 PUFA in the P/S ratio 2.0 diet, the associated changes in phospholipid fatty acid composition were essentially limited to modest increases in 18:2, n-6 and in the n-6:n-3 ratios. Levels of 20:4, n-6 were unaltered in relation to the n-6 PUFA diet, indeed in PE there was a small decrease in this fatty acid species. It may therefore be suggested that the n-6 PUFA provided by this diet was insufficient to influence fatty acid composition and perhaps vascular responses. However, although we did not examine the effects of diets containing pure sunflower seed oil or safflower seed oil (sources of n-6 PUFA), a P/S ratio of 2.0 in a 40% fat calorie diet is the maximum that can be achieved without resorting to liquid formulation and we wished to adhere to diets that could be consumed by man.

The significant change in response to α_1-adrenoceptor stimulation following dietary n-3 PUFA took the form of an almost parallel depression of the rise in perfusion pressure. This phenomenon suggests that, although baseline pressure was not influenced by diet, some enhancement of vascular relaxation may have contributed to the observed effects. It is recognised that dietary n-3 PUFA can facilitate endothelium-dependent vascular relaxation [Shimokawa *et al.*, 1987] and this has been shown to apply to acetylcholine mediated relaxation of isolated rat aortic tissue [Yin *et al.*, 1988, Yin *et al.*, 1991, Malis *et al.*, 1991]. In our study, the vasoconstricted hind-

quarters was seen to vasodilate to acetylcholine as a qualitative means of assessing endothelial integrity but it was not part of the study to document this response in a controlled quantitative manner. Accordingly, an effect of our n-3 PUFA diet on the relaxant state of the perfused vessels cannot be excluded.

We emphasise that two separate studies were performed in two separate strains at different times. Seasonal influences on the animals are not relevant under controlled environmental conditions but nevertheless, differences between the control groups might have contributed to the apparently contrasting effects of n-3 and n-6 PUFA. Although the n-3 and n-6 PUFA studies were not contemporaneous, comparison of the perfusion pressure responses of the two control groups did not reveal any significant differences. However, analysis of variance applied to the phospholipid fatty acid composition of the two control groups confirmed that they varied, notably so for n-6 fatty acid species in PC and 22:6, n-3 in PI. Whether these differences could have influenced the results needs to be clarified by experiments designed around the simultaneous administration of the three diets.

To summarise, supplementation of a 40% fat calorie diet with a small amount of n-3 PUFA was found to attenuate vascular responses to α_1-adrenoceptor stimulation in the rat hind-quarters perfused at constant flow. In the following chapter, the possibility that dietary n-3 PUFA alters both vascular contraction and relaxation is pursued in an isometric myograph model, and the problem of differing control groups and strains is resolved to allow direct comparison of control, n-3 PUFA and n-6 PUFA diets.

Chapter 4

Dietary polyunsaturated fatty acids and the contraction and relaxation of resistance arteries

4.1 Introduction

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In the hind-quarters of the rat perfused at constant flow, we demonstrated that supplementation of the diet with a small dose of n-3 PUFA brought about the attenuation of responses to α_1-adrenoceptor stimulation. Changes in phospholipid fatty acid composition in relation to dietary n-3 PUFA suggested a number of potential mechanisms. It was also possible that differences in vascular relaxant state between the control and n-3 PUFA groups could have contributed to the findings. A second separate study did not reveal any significant effect of dietary n-6 PUFA on α_1-adrenoceptor mediated responses. These two dietary PUFA studies were performed with different strains of rats. Although the two control groups did not differ in vascular responses, they did differ in phospholipid fatty acid composition, further precluding true comparison of the n-3 PUFA and n-6 PUFA effects.

The principal effectors of the vasoconstrictor response to α_1-adrenoceptor stimulation in the peripheral vascular bed are distal resistance arteries rather than proximal conduit vessels. In order to clarify the effects of n-3 PUFA on vascular reactivity and to facilitate the investigation of the possible effects of dietary PUFA on vascular relaxation, we decided to direct our attention to the isolated resistance artery. Using an isometric myograph, which allows different vessels to be maintained at physiologically equivalent degrees of tension, we studied the effect of dietary PUFA

on contraction and relaxation in second order femoral artery branches isolated from the rat. Once again, the control diet derived 40% of dietary calories from fat and had a P/S ratio of 0.3. Supplementation of this diet with 0.4% calories fish oil provided the n-3 PUFA diet. A diet of P/S ratio 2.0, relatively rich in 18:2, n-6, constituted the n-6 PUFA diet.

4.2 Methods

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Thirty Sprague-Dawley rats were separated at random into three groups under the conditions described in section 2.1. Semisynthetic experimental diets were fed for eight weeks. The n-3 PUFA group received a daily oral dose of fish oil in olive oil and the control and n-6 PUFA groups received daily oral doses of olive oil alone. As described in section 2.2.4, the olive oil administered to the three groups was not supplementary to dietary requirement. The overall dietary composition is shown in Table 4.1. At the end of the feeding period, the animals were sacrificed and the surgical procedures and protocol of sections 2.5.3 and 2.5.4 were carried out.

TABLE 4.1 *Calculated fat composition (%) of the experimental diets.*

	Diet		
Fatty acid classes	Control	n-3 PUFA	n-6 PUFA
Saturates	20%	20%	8.7%
Monoenes	14%	14%	14%
n-6 PUFA	6%	6%	17.3%
n-3 PUFA	-	0.4%	-
Total	40%	40.4%	40%

Values represent the proportion of dietary calories supplied by different classes of fatty acid.

Of the 60 vessels dissected and mounted, 8 were excluded on the basis of either an effective internal diameter exceeding 300 μm or the inability to contract against a

tension equivalent to a transmural pressure of 100 mm Hg (control group: 1, n-3 PUFA group: 4, n-6 PUFA group: 3). Contractions to noradrenaline 3 nM-10 μM were elicited in the presence of yohimbine 1 μM and timolol 1 μM. Subsequently, we recorded the relaxation to acetylcholine 1 nM-0.1 mM of vessels pre-constricted (with noradrenaline 0.3 μM or 1 μM) to approximately 60% of their previous maximum contraction. One vessel from the control group was found to contract further (+ 20%) to low doses of acetylcholine, was presumed de-endothelialised and was excluded from further analysis. Subsequently, for statistical purposes, 3 vessels (control group: 2, n-6 PUFA group: 1) were excluded at random to provide balanced groups of 16 vessels. Analysis of the data by rat (3 groups of 8) did not provide different results. The final groups did not differ with respect to L_{100} values (see section 2.5.4) or vessel segment length (Table 4.2).

TABLE 4.2 *L_{100} values (μm) and vessel segment length (mm) of femoral resistance arteries isolated from rats fed experimental diets.*

Diet	L_{100}	Segment lengths
Control	243±9	2.10±0.07
n-3 PUFA	257±8	2.10±0.06
n-6 PUFA	245±7	2.00±0.10

Values: mean ± s.e.m., n=16 per dietary group. There were no significant differences between the groups.

4.3 Results

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4.3.1 Dietary n-3 PUFA and contractile responses of the resistance artery

Noradrenaline in the presence of yohimbine and timolol evoked considerable increases in active tension. Vessels isolated from rats fed the n-3 PUFA diet demonstrated significantly attenuated responses compared to control ($p< 0.01$, ANOVA-RM, Fig. 4.1). The effect was evident at higher concentrations of agonist and involved a

depression of gradient and maximal response. At any specific concentration of noradrenaline, however, there was no significant difference in response in relation to diet.

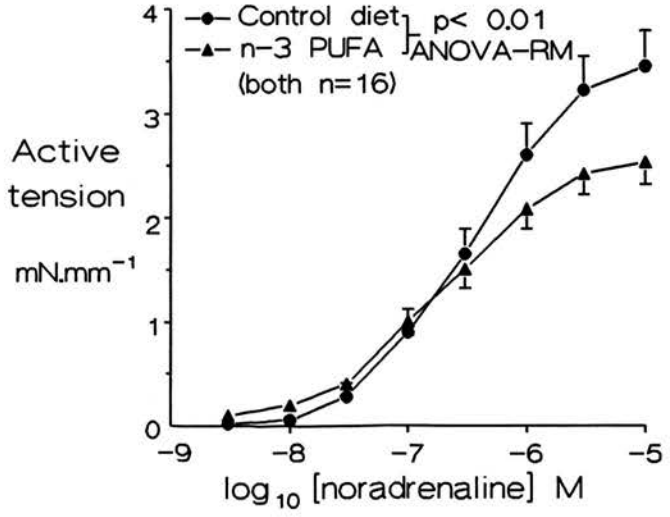

FIGURE 4.1 *Changes in active tension (mN.mm^{-1}) in response to noradrenaline (3 nM-10 μM), in the presence of yohimbine (1 μM) and timolol (1 μM), in resistance arteries isolated from rats fed experimental diets: n-3 PUFA.*

4.3.2 Dietary n-6 PUFA and contractile responses of the resistance artery

Vessels isolated from rats fed the n-6 PUFA diet showed a downward trend in contractile response compared to control throughout most of the dose response relationship but this did not achieve statistical significance (Fig. 4.2).

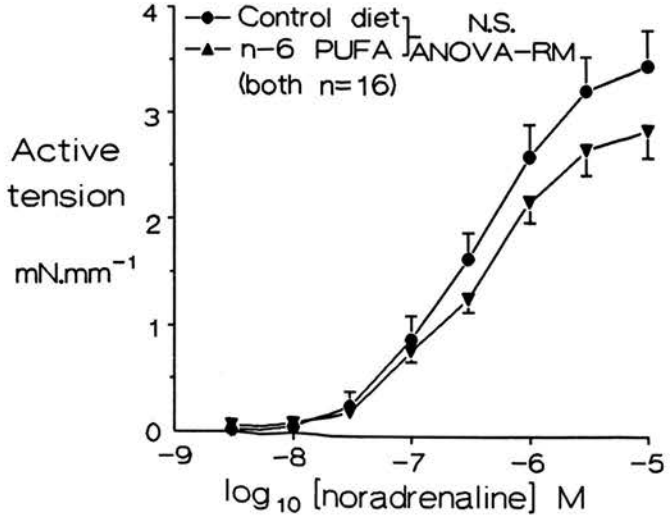

FIGURE 4.2 *Changes in active tension (mN.mm^{-1}) in response to noradrenaline (3 nM-10 μM), in the presence of yohimbine (1 μM) and timolol (1 μM), in resistance arteries isolated from rats fed experimental diets: n-6 PUFA.*

4.3.3 Dietary PUFA and relaxation of the resistance artery

Neither the n-3 PUFA diet nor the n-6 PUFA diet exerted any significant effect on the relaxation of pre-constricted vessels in response to acetylcholine (Fig. 4.3).

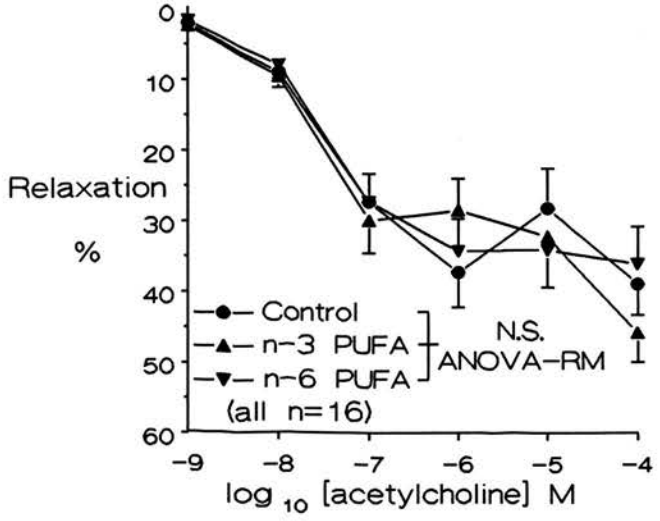

FIGURE 4.3 *Relaxation (%) of pre-constricted isolated resistance arteries in response to acetylcholine (1 nm-0.1 mM).*

Prior to relaxation, the vessels of the three dietary groups were constricted to the same degree (Table 4.3).

TABLE 4.3 *Active tension ($mN.mm^{-1}$) of isolated resistance arteries pre-constricted with noradrenaline 0.3 μM or 1 μM.*

Diet	Active tension
Control	2.3±0.2
n-3 PUFA	2.1±0.2
n-6 PUFA	2.1±0.2

Values: mean ± s.e.m., n=16 per dietary group. There were no significant differences between the groups.

4.3.4 Body weights

The final body weights for animals fed the n-6 PUFA diet were significantly greater than those of animals fed the n-3 PUFA diet but neither differed significantly from

control. Inspection of the weights at arrival confirmed that the groups had varied at the outset and, although weight gain tended to be greater in the n-3 PUFA group, there were no significant differences (Table 4.4).

TABLE 4.4 *Body weights (g) and weight gain (g) of rats fed experimental diets.*

Diet	Initial weight	Final weight	Weight gain
Control	220±9[a]	379±8	159±5
n-3 PUFA	189±2[a,b]	363±6[c]	174±8
n-6 PUFA	240±3[b]	394±8[c]	154±7

Values: mean ± s.e.m., n=16 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) with respect to diet.

4.3.5 Fatty acid composition of adipose tissue

The n-3 PUFA diet, as expected, did not influence adipose tissue 18:2, n-6 or 20:4, n-6. The n-3 fatty acid species 20:5, n-3 was not detected (< 0.1%) and 22:6, n-3 was only detected in relation to n-3 PUFA supplementation. Highly significant increases in 18:2, n-6 and in the small proportion of 20:4, n-6 were seen in relation to the P/S 2.0 diet, confirming consumption (Table 4.4).

TABLE 4.5 *Fatty acid composition of adipose tissue from rats fed experimental diets.*

	Diet		
Fatty acid species	Control	n-3 PUFA	n-6 PUFA
18:2, n-6	10.6±0.5[a]	11.6±0.5[b]	32.3±0.4[a,b]
20:4, n-6	0.1±0.0[c]	0.2±0.0[d]	0.5±0.1[c,d]
20:5, n-3	-	-	-
22:6, n-3	-	0.1±0.0	-

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. Superscript characters denote significant differences ($p < 0.01$) between individual fatty acid species with respect to diet.

4.3.6 Fatty acid composition of cardiac total and fractionated phospholipid

The absolute amounts of total phospholipid and of the individual fractions (Table 4.6) were lower than is usually found with these 40% fat calorie diets in our laboratory (see Tables 3.7, 3.11, 6.9, 7.8 and 8.2) but the relative proportions of the individual phospholipid fractions were similar to these other studies. There was no significant dietary effect.

TABLE 4.6 *Total phospholipid and phospholipid fractions in hearts of rats fed experimental diets.*

Phospholipid	Diet		
fraction	Control	n-3 PUFA	n-6 PUFA
PC	3757±227	3872±205	4428±276
PE	2951±366	2908±205	2773±275
PI	408±38	401±21	463±23
PS	257±8	237±13	237±21
Total	8384±861	9548±752	10022±371

Values (mean ± s.e.m.) are expressed as μg.g wet heart weight^{-1}, n=8 per dietary group. There were no significant differences between the groups.

The analysis of the fatty acid composition of total and fractionated phospholipid again revealed numerous statistically significant differences with respect to diet. In total phospholipid (Table 4.7), supplementation of the diet with n-3 PUFA led to small reductions in the proportions of 18:2, n-6 and 20:4, n-6 and to an increase in the minor proportion of 20:5, n-3. There was a marked increase in 22:6, n-3. The n-6:n-3 ratio was halved and the double bond index rose significantly. The P/S ratio 2.0 diet was associated with a limited increase in 18:2, n-6 and did not affect 20:4, n-6. With a reduction of a third in the level of 22:6, n-3, the n-6:n-3 ratio was increased 50% but the double bond index fell.

TABLE 4.7 *Fatty acid composition of total phospholipid, including the n-6:n-3 ratio and double bond index (DBI), in the hearts of rats fed experimental diets.*

Fatty acid species,	Diet		
n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
18:2, n-6	18.4 ± 1.4^{a}	16.4 ± 2.7^{b}	$21.0\pm1.3^{a,b}$
20:4, n-6	23.9 ± 1.0^{c}	$20.0\pm1.0^{c,d}$	23.3 ± 1.4^{d}
20:5, n-3	0.1 ± 0.1^{e}	0.5 ± 0.2^{e}	-
22:6, n-3	$9.9\pm0.5^{f,g}$	$17.5\pm2.4^{f,h}$	$6.9\pm0.8^{g,h}$
n-6:n-3 ratio	$4.2\pm0.5^{i,j}$	$1.9\pm0.5^{i,k}$	$6.4\pm1.0^{j,k}$
DBI	$230\pm2^{l,m}$	$240\pm1^{l,n}$	$224\pm1^{m,n}$

Values: mean ± s.e.m., n=8 per dietary group. Data for fatty acids represent the percentage weight of total fatty acid species. Superscript characters denote significant differences ($0.001< p <0.05$) between individual fatty acid species and parameters with respect to diet.

The fatty acid composition of fractionated phospholipid (Table 4.8) revealed that the decreases in 18:2, n-6 and 20:4, n-6 brought about by the n-3 PUFA diet were confined to PC and PE, being especially marked in the latter. There was a small increase in the minor proportion of 20:5, n-3 in PE and this fatty acid species was only detected in PC in relation to n-3 PUFA supplementation; 20:5, n-3 was not detected in PI or PS (< 0.1%). Increases in 22:6, n-3 ranged from 30% in PS to 200% in PE. The n-6 PUFA diet led to modest increases in 18:2, n-6 in all fractions bar PS, but failed to influence levels of 20:4, n-6, except in PE where, as noted in the previous chapter, there was a small but significant decrease. The proportion of 22:6, n-3 fell by 25% in PC, PE and PS in relation to n-6 PUFA feeding but was not affected in PI. The most striking finding in the analysis of fractionated phospholipid fatty acid composition was the close conservation of 20:4, n-6 in PI regardless of diet.

TABLE 4.8 *Fatty acid composition of phospholipid fractions in the hearts of rats fed experimental diets.*

Phospholipid fraction and fatty acid species		Diet		
		Control	n-3 PUFA	n-6 PUFA
PC	18:2, n-6[c]	9.1±0.4	7.4±0.7	10.3±0.5
	20:4, n-6[a,c]	29.7±0.6	25.8±0.4	30.2±0.5
	20:5, n-3	-	0.6±0.1	-
	22:6, n-3[a,b,c]	4.1±0.1	9.6±0.8	2.9±0.2
PE	18:2, n-6[b,c]	4.6±0.1	3.8±0.3	6.5±0.3
	20:4, n-6[a,b,c]	25.9±0.4	18.5±1.0	23.2±0.5
	20:5, n-3[a,b,c]	0.2±0.0	0.5±0.1	0.1±0.0
	22:6, n-3[a,b,c]	20.2±0.2	32.9±1.5	15.2±0.7
PI	18:2, n-6[b,c]	4.2±0.2	4.2±0.2	7.5±0.4
	20:4, n-6	35.5±0.9	34.8±1.0	33.6±1.1
	20:5, n-3	-	-	-
	22:6, n-3[a,c]	1.5±0.1	4.0±0.3	1.4±0.4
PS	18:2, n-6	2.6±0.3	1.9±0.2	2.8±0.1
	20:4, n-6	10.3±0.6	9.0±0.5	11.4±0.3
	20:5, n-3	-	-	-
	22:6, n-3[a,b,c]	19.0±0.9	25.1±1.3	13.6±0.3

Values are means ± s.e.m. and represent the percentage weight of total fatty acid species within each phospholipid fraction, n=8 hearts per dietary group. Significant differences ($0.001 < p < 0.05$) between individual fatty acid species with respect to diet are denoted by superscript characters. [a] control *vs* n-3 PUFA, [b] control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA.

In response to n-3 PUFA supplementation, the n-6:n-3 ratios in all fractions were halved and the double bond index increased significantly in PC and PE, though not in PI and PS (Table 4.9). The n-6 PUFA diet increased the n-6:n-3 ratios by approximately 50% but did not significantly affect the double bond indices.

TABLE 4.9 *Phospholipid fraction n-6:n-3 ratios and double bond indices (DBI) in the hearts of rats fed experimental diets.*

Phospholipid fraction,		Diet		
	n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
PC	n-6:n-3 ratio[a,b,c]	7.7±0.3	2.9±0.4	11.8±0.9
	DBI[a,c]	187±1	197±2	186±2
PE	n-6:n-3 ratio[a,b,c]	1.6±0.0	0.7±0.1	2.5±0.1
	DBI[a,c]	280±2	296±2	276±2
PI	n-6:n-3 ratio[a,c]	1.5±0.1	4.0±0.3	1.4±0.4
	DBI	184±3	186±4	184±4
PS	n-6:n-3 ratio[a,b,c]	1.1±0.1	0.6±0.0	2.1±0.0
	DBI	188±4	186±5	182±4

Values: mean ± s.e.m., n=8 per dietary group. Superscript characters denote significant differences ($0.0001 < p < 0.05$) between parameters for individual phospholipid fractions in relation to diet. [a] control *vs* n-3 PUFA, [b] control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA.

4.4 Discussion

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The contractile responses of isolated rat femoral resistance arteries were attenuated by supplementation of a 40% fat calorie diet of P/S ratio 0.3 with a small daily dose of n-3 PUFA. Increasing the n-6 PUFA content of the diet to a P/S ratio of 2.0 did not significantly affect contractile responses although a downward trend was evident. In this experimental model, contractions were elicited by noradrenaline in the presence of yohimbine and timolol, thus we infer that these responses were mediated by α_1-adrenoceptors. Neither of the PUFA diets had any influence on the acetylcholine induced relaxation of pre-constricted vessels. The fatty acid composition of these small vessels could not be determined due to the amounts of tissue required for analysis. The effect of the diets was therefore monitored in cardiac tissue. Both PUFA diets were

associated with significant changes in cardiac fractionated phospholipid fatty acid composition, changes which were more extensive in relation to dietary n-3 PUFA.

The effect of dietary n-3 PUFA on the contractile responses of isolated resistance arteries is consistent with our observations in the perfused hind-quarters of the rat (Chapter 3). In the present study, the effect was apparent at higher doses of agonist, a phenomenon also reported in rat aortic rings [Yin *et al.*, 1991]. The findings are also consistent with other results obtained in the rat aorta following higher doses of n-3 PUFA [Lockette *et al.*, 1982, Malis *et al.*, 1991] to which we referred in Chapter 3.

The changes observed in cardiac fractionated phospholipid fatty acid composition in relation to the n-3 PUFA diet were very similar to those described in Chapter 3. Once again, if these changes occurred in the resistance arteries, the increased n-3 fatty acid species content, increased unsaturation and decreased 20:4, n-6 content of PC and PE may have led to the attenuation of contraction. It was again evident that the fatty acid composition of the important PI fraction was relatively resistant to the influence of dietary n-3 PUFA, particularly with respect to 20:4, n-6.

The diet rich in n-6 PUFA did not exert any significant effect on the contractile responses of the isolated resistance arteries but, in contrast to the results in the perfused rat hind-quarters, there was an obvious trend toward attenuation. This trend might be important but potential mechanisms are obscure. There were some statistically significant changes in fractionated phospholipid fatty acid composition in relation to the n-6 PUFA diet but these were limited. Further, although n-6:n-3 ratios rose, the double bond indices did not differ from control and were significantly lower than for dietary n-3 PUFA in PC and PE. Increased release of prostacyclin or other vasodilator prostaglandins, not investigated in our study, would be an attractive explanation for an attenuatory effect of n-6 PUFA on contractile responses. However, a mechanism of this nature is difficult to support as levels of precursor 20:4, n-6 were unaltered in PC, PI and PS and actually fell in PE following the n-6 PUFA diet.

The lack of any obvious potential mechanism underlying the n-6 PUFA trend renders the whole study less easy to interpret. Whereas the n-3 and n-6 PUFA effects

on contraction were in the same direction; significant attenuation for the former and non-significant attenuation for the latter, this was not true for their effects on fatty acid composition. The significant effects of dietary n-6 PUFA on fatty acid composition were generally opposite in direction to those of n-3 PUFA. Accordingly, the mechanisms suggested above for n-3 PUFA are unlikely to be direct or linear.

In Chapter 3, we suggested that enhanced relaxation of the perfused vessels in the n-3 PUFA group could have contributed to the findings. In the present study, the normalisation procedure of the isometric myograph technique (section 2.5.4), which sets different vessels to physiologically equivalent degrees of stretch, should minimise baseline variations in relaxation state [Dainty *et al.*, 1990]. It is interesting therefore that the contractile responses of resistance arteries in the n-3 PUFA group overlap those of the control group during the initial part of the dose response relationship whereas the effect of n-3 PUFA was present throughout in the perfused hind-quarters. This would be consistent with a baseline effect of n-3 PUFA in the hind-quarters model which was not seen following normalisation in the myograph model. However, it would follow that the vessels from the n-3 PUFA group would require a greater degree of stretch to bring them to the same level as control and n-6 PUFA vessels and this was not so; L_{100} was not affected by diet. Thus, although the pattern of n-3 PUFA effect in the two preparations varies, this is unlikely to be due to removal of an important baseline effect of n-3 PUFA in the isolated resistance artery.

Neither PUFA diet affected relaxation of the pre-constricted vessels to acetylcholine. With regard to n-3 PUFA, this contradicts reports of enhanced acetylcholine induced relaxation of aortic rings isolated from rats fed fish oil [Yin *et al.*, 1988, Malis *et al.*, 1991] and is inconsistent with the enhanced endothelium-dependent relaxation of coronary artery rings from swine fed fish oil [Shimokawa *et al.*, 1987]. There are two points. First, we used a small dose of fish oil. The effect of fish oil on the swine coronaries was shown to be dose dependent and relaxation was much more pronounced with doses of n-3 PUFA 2 to 3-fold greater than in our study. Second, there is recent evidence of regional variation in vascular relaxation responses

in the rat. Enhanced acetylcholine induced relaxation of rat aortic rings in relation to dietary n-3 PUFA was not apparent in mesenteric resistance arteries isolated from the same animals, arteries which had nevertheless demonstrated an attenuated response to noradrenaline [Yin *et al.*, 1991]. Nagao *et al.* [1992] have proposed that endothelium-dependent relaxations in vessels of small calibre differ from those of large calibre.

In summary, a small dose of dietary n-3 PUFA attenuated the contractile responses of isolated resistance arteries to α_1-adrenoceptor stimulation. Associated changes in the fatty acid composition of fractionated phospholipid suggested certain mechanisms but there were contradictions in the effects of dietary n-6 PUFA. The following chapter describes our initial studies of the effects of dietary PUFA on α_1-adrenoceptor stimulated responses in the isolated heart.

Chapter 5

Polyunsaturated fatty acids in 10% fat calorie diets and the heart

5.1 Introduction

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The effects of dietary PUFA on the heart are the focus of much attention, particularly with regard to n-3 PUFA. However, relatively few reports concern dietary PUFA and aspects of cardiac physiology. Of these reports, the majority disagree over whether n-6 PUFA exert positive or negative inotropic effects in various experimental models. Recently, it was reported that dietary menhaden oil (relatively rich in n-3 PUFA), but not corn oil (rich in n-6 PUFA), attenuated the response of $dP.dt^{-1}_{max}$ to α_1-adrenoceptor stimulation in retrogradely perfused isolated rat hearts [Reibel *et al.*, 1988]. These n-3 and n-6 PUFA diets derived 12% of total calories from fat. In the same study, responses to isoprenaline were not affected. We found this report particularly interesting in view of our findings in the perfused rat hind-quarters (Chapter 3) and isolated rat resistance artery (Chapter 4) but felt that certain questions should be addressed. First, the n-3 PUFA diet used was probably deficient in n-6 essential fatty acids and the control diet was actually a laboratory chow, low in fat (5% dietary calories). Second, work with isolated rabbit papillary muscles [Dukes and Vaughan Williams, 1984] and isolated rabbit hearts [Aoyagi *et al.*, 1991] suggests that changes in peak LVP rather than $LVdP.dt^{-1}$ would better reflect α_1-adrenoceptor

stimulation in the isolated heart. Third, there was the possibility that dietary PUFA might have influenced neuronal noradrenaline content and release [Wince *et al.*, 1981, Semafuko *et al.*, 1987, Semafuko *et al.*, 1989] and thus could have affected the results.

We therefore decided to examine LV responses to α_1-adrenoceptor stimulation (noradrenaline in the presence of timolol) in rats fed isocalorific semisynthetic diets deriving 10% of dietary calories from fat. The control diet had a P/S ratio of 0.3. To prevent essential fatty acid deficiency, the n-3 PUFA diet contained sufficient 18:2, n-6. This was also the predominant fatty acid in the n-6 PUFA diet. In a second related study, we investigated the effect of chemical denervation on the responses of hearts from rats fed the two PUFA diets.

5.2 Methods

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5.2.1 Dietary PUFA and cardiac α_1-adrenoceptor responses

Thirty-six Sprague-Dawley rats were separated at random into three groups of 12 under the conditions described in section 2.1. The precautions discussed in sections 2.2.2. and 2.2.3 were taken in the administration of the 10% fat calorie diets (Table 5.1). After an eight week feeding period, the surgical procedures of section 2.6.3 were performed. Hearts were perfused according to the protocol of section 2.6.4. After stabilising, the responses of the isolated perfused hearts to noradrenaline 0.1-2.4 μM in the presence of timolol 10 μM were recorded. Five hearts (control: 2, n-3 PUFA: 1, and n-6 PUFA: 2) were excluded according to the criteria given in the protocol.

TABLE 5.1 *Calculated fatty acid composition (%) of the control, n-3 PUFA and n-6 PUFA experimental diets.*

	Diet		
Fatty acid classes	Control	n-3 PUFA	n-6 PUFA
Saturates	5.0%	2.3%	1.5%
Monoenes	3.5%	2.2%	3.3%
n-6 PUFA	1.5%	2.5%	5.2%
n-3 PUFA	-	3.0%	-
Total	10%	10%	10%

Percentage values reflect the proportion of dietary calories supplied by different classes of fatty acid.

5.2.2 Influence of chemical denervation

For the second study, 30 Sprague-Dawley rats were separated at random into two groups of 15. One group received the n-3 PUFA 10% fat calorie diet and the other the n-6 PUFA diet. After five weeks feeding, the animals began the intraperitoneal 6-hydroxy-dopamine regimen of section 2.6.6. At the end of eight weeks feeding, an experiment identical to the above was performed. No experimental exclusions were indicated. In order to assess the effect of the 6-hydroxy-dopamine regimen, denervated and non-denervated hearts (from the previous experiment) were assayed for noradrenaline content (section 2.7.6).

5.3 Results

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5.3.1 Dietary PUFA and LVP

An effect of dietary PUFA on peak LVP in the absence of noradrenaline infusion was evident. The hearts from the n-3 PUFA group maintained significantly lower tensions

than hearts from the control and n-6 PUFA groups, which were identical to one another (Table 5.1).

TABLE 5.2 *Peak LVP (mmHg) prior to noradrenaline infusion, in the presence of timolol 10 μM, in hearts isolated from rats fed experimental diets.*

Diet	LVP
Control	84 ± 6^{a}
n-3 PUFA	$67\pm3^{a,b}$
n-6 PUFA	84 ± 6^{b}

Values: mean ± s.e.m., n=10 per dietary group. Superscript characters denote significant differences ($p< 0.05$).

A similar pattern was seen in the dose response relationships to noradrenaline where the n-3 PUFA diet was associated with significant parallel attenuation of the peak LVP responses to α_1-adrenoceptor stimulation compared to the control diet ($p< 0.05$, ANOVA-RM, Fig. 5.1). As indicated, secondary analysis identified significant differences at the lower three concentrations of agonist ($p< 0.05$, *t* test).

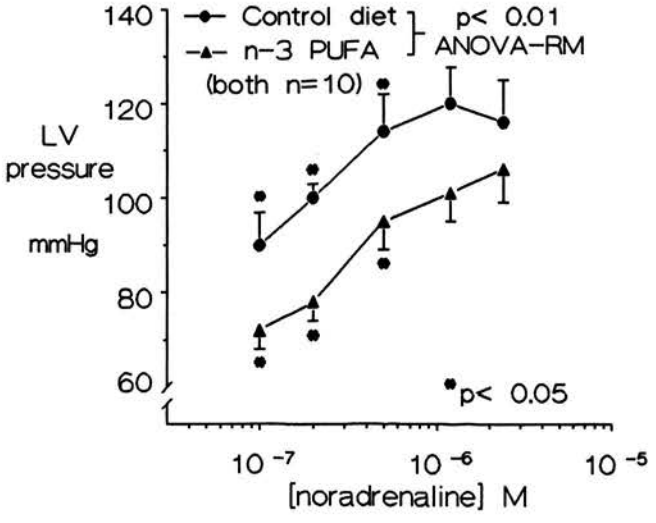

FIGURE 5.1 *Response of peak LVP (mm Hg) to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets: n-3 PUFA.*

The n-6 PUFA diet was associated with slightly increased responses which did not differ significantly from control (Fig. 5.2).

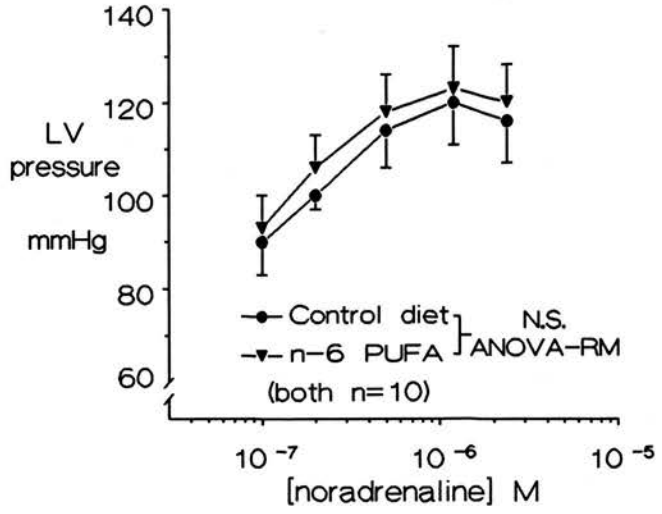

FIGURE 5.2 *Response of peak LVP (mm Hg) to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets: n-6 PUFA.*

Comparison of the two PUFA groups confirmed that they differed overall ($p < 0.05$, ANOVA-RM, Fig. 5.3) and at the lower four concentrations of noradrenaline ($p < 0.05$, *t* test).

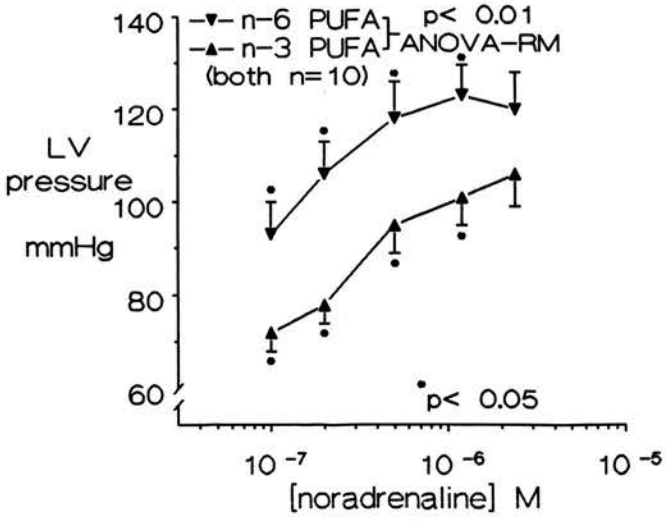

FIGURE 5.3 *Response of peak LVP (mm Hg) to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets: n-3 PUFA and n-6 PUFA.*

5.3.2 Dietary PUFA and $LVdP.dt^{-1}$

TABLE 5.3 *$LVdP.dt^{-1}_{max}$ ($mmHg.sec^{-1}$) at baseline and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets.*

Noradrenaline concentration	Diet		
	Control	n-3 PUFA	n-6 PUFA
Baseline	3060±180	2840±180	3380±420
0.10 μM	3220±240	3310±270	3640±340
0.19 μM	3420±220	3520±250	4280±450
0.52 μM	3840±220	4080±280	4500±350
1.22 μM	4080±250	4160±280	4580±330
2.37 μM	3960±240	4140±300	4640±330

Values: mean ± s.e.m., n=10 per dietary group. There were no significant differences between dietary groups.

TABLE 5.4 *$LVdP.dt^{-1}_{min}$ ($mmHg.sec^{-1}$) at baseline and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets.*

Noradrenaline concentration	Diet		
	Control	n-3 PUFA	n-6 PUFA
Baseline	1733±210	1475±427	1771±150
0.10 μM	1967±240	1675±170	2143±310
0.19 μM	2267±290	1850±160	2514±300
0.52 μM	2667±260	2150±180	2971±340
1.22 μM	2700±300	2275±190	3029±280
2.37 μM	2533±240	2350±210	2914±340

Values: mean ± s.e.m., n=10 per dietary group. There were no significant differences between dietary groups.

LVdP.dt^{-1} increased in all three dietary groups with α_1-adrenoceptor stimulation. Neither the responses of dP.dt$^{-1}_{max}$ (Table 5.2) nor dP.dt$^{-1}_{min}$ (Table 5.3) were significantly influenced by diet (ANOVA-RM, N.S.).

5.3.3 Dietary PUFA and heart rate

Resting heart rates were in close agreement. There was a tendency toward initial bradycardia in all three groups but this was not significant (Table 5.6). Neither n-3 nor n-6 PUFA significantly affected the heart rate responses to α_1-adrenoceptor stimulation (ANOVA-RM, N.S.).

TABLE 5.6 *Rates (bpm) at rest and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), of hearts isolated from rats fed experimental diets.*

Noradrenaline concentration	Diet: Control	n-3 PUFA	n-6 PUFA
Baseline	269±10	279±10	275±9
0.10 μM	260±7	256±9	256±4
0.19 μM	251±5	245±7	260±8
0.52 μM	255±4	252±4	255±4
1.22 μM	264±5	255±6	263±7
2.37 μM	278±7	254±7	270±5

Values: mean ± s.e.m., n=10 per dietary group. There were no significant differences between dietary groups.

5.3.4 Dietary PUFA: coronary flow and lactate production

Neither baseline coronary flow nor lactate production varied with respect to diet (Table 5.7, overleaf).

TABLE 5.7 *Resting coronary flow (ml.min^{-1}.g wet heart weight^{-1}) and lactate production (μmol.min^{-1}.g wet heart weight^{-1}) of hearts isolated from rats fed experimental diets.*

Diet	Flow	Lactate
Control	9.4±0.5	0.41±0.08
n-3 PUFA	9.6±0.3	0.41±0.06
n-6 PUFA	9.2±0.2	0.52±0.07

Values: mean ± s.e.m., n=10 per dietary group. There were no significant differences between dietary groups.

5.3.5 Dietary PUFA: body and heart weights

The final body weights and wet heart weights were similar in the three dietary groups (Table 5.8).

TABLE 5.8 *Final body weights (g) and wet heart weights (g) of rats fed experimental diets.*

Diet	Body weight	Heart weight
Control	350±9	1.06±0.02
n-3 PUFA	354±8	1.09±0.03
n-6 PUFA	360±8	1.08±0.03

Values: mean ± s.e.m., n=10 per dietary group. There were no significant differences between the groups.

5.3.6 Fatty acid composition of adipose tissue

Both the n-3 PUFA and n-6 PUFA diets brought about statistically significant changes in adipose fatty acid composition (Table 5.9). The greater proportion of 18:2, n-6 and a small but significant increase in the minor proportion of 20:4, n-6 in relation to the n-3 PUFA diet compared to control reflected the composition of the diets (see Table 5.1). The n-3 fatty acid species 20:5, n-3 and 22:6, n-3 were only detectable in animals

fed the n-3 PUFA diet. The n-6 PUFA diet led to 4-fold increases in 18:2, n-6 and 20:4, n-6.

TABLE 5.9 *Fatty acid composition of adipose tissue from rats fed experimental diets.*

	Diet		
Fatty acid species	Control	n-3 PUFA	n-6 PUFA
18:2, n-6	6.6±0.4[a,b]	14.0±0.7[a,c]	27.1±0.9[b,c]
20:4, n-6	0.1±0.0[d,e]	0.3±0.0[d]	0.4±0.0[e]
20:5, n-3	-	1.6±0.1	-
22:6, n-3	-	1.9±0.1	-

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. Superscript characters denote significant differences ($p < 0.001$) between individual fatty acid species in relation to diet.

5.3.7 Fatty acid composition of cardiac total and fractionated phospholipid

The PUFA diets did not affect the amount of total phospholipid or the contribution of the individual phospholipid fractions (Table 5.10). Analysis of the fatty acid composition of cardiac total and fractionated phospholipid revealed numerous statistically significant differences. The n-3 PUFA diet brought about moderate increases in 18:2, n-6 in total phospholipid (Table 5.11) compared to the control diet but 20:4, n-6 was more than halved. There was a substantial increase in the small proportion of 20:5, n-3 with this diet and 22:6, n-3 tripled. The n-6:n-3 ratio fell 4-fold and the double bond index increased significantly. Although the n-6 PUFA diet raised the level of 18:2, n-6 relative to control, it did not exceed that of the n-3 PUFA diet. Paradoxically, 20:4, n-6 was lower in relation to the n-6 PUFA diet than to control. Of the n-3 fatty acid species, 20:5, n-3 was not detected (< 0.1%) and 22:6, n-3 was 60% that of control and 20% that of the n-3 PUFA diet. The n-6:n-3 ratios nearly doubled with n-6 PUFA but there was no significant change in the double bond index.

TABLE 5.10 *Total phospholipid and phospholipid fractions in hearts isolated from rats fed experimental diets.*

Phospholipid	Diet		
fraction	Control	n-3 PUFA	n-6 PUFA
PC	3987±181	4276±249	4101±207
PE	3364±156	3178±141	3248±179
PI	450±20	443±17	421±19
PS	251±11	238±14	245±17
Total	9974±236	10093±210	11162±244

Values (mean ± s.e.m.) are expressed as μg fatty acids.g wet heart weight^{-1}, n=8 per dietary group. There were no significant differences between the groups.

TABLE 5.11 *Fatty acid composition of total phospholipid, including the n-6:n-3 ratio and double bond index (DBI), in hearts isolated from rats fed experimental diets.*

Fatty acid species,	Diet		
n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
18:2, n-6	$20.8\pm1.3^{a,b}$	24.5 ± 0.6^{a}	25.5 ± 1.0^{b}
20:4, n-6	$21.8\pm0.4^{c,d}$	$10.1\pm0.2^{c,e}$	$18.5\pm0.5^{d,e}$
20:5, n-3	0.2 ± 0.0^{f}	1.7 ± 0.1^{f}	-
22:6, n-3	$5.2\pm0.4^{g,h}$	$15.6\pm0.4^{g,i}$	$3.0\pm0.3^{h,i}$
n-6:n-3 ratio	$8.0\pm0.7^{j,k}$	$1.8\pm0.1^{j,l}$	$15.3\pm1.2^{k,l}$
DBI	200 ± 3^{m}	$216\pm2^{m,n}$	195 ± 2^{n}

Values: mean ± s.e.m., n=8 per dietary group. Data for fatty acids represent the percentage weight of total fatty acid species. Superscript characters denote significant differences ($0.001< p < 0.05$) between individual fatty acid species and parameters in relation to diet.

In the phospholipid fractions (Table 5.12), the n-3 PUFA diet was associated with slightly higher levels of 18:2, n-6 than control but there were decreases in 20:4, n-6 ranging from 30% in PC and PI to 70% in PE. Of the n-3 fatty acids, 20:5, n-3 was

present in small amounts in all fractions and marked increases in 22:6, n-3 were confined to PC, PE and PI.

TABLE 5.12 *Fatty acid composition of phospholipid fractions in hearts isolated from rats fed experimental diets.*

Phospholipid fraction and fatty acid species		Diet		
		Control	n-3 PUFA	n-6 PUFA
PC	18:2, n-6[a,b]	10.8±0.4	14.4±0.8	13.8±0.7
	20:4, n-6[a,c]	22.4±0.7	13.5±0.3	22.5±0.8
	20:5, n-3	-	2.2±0.1	-
	22:6, n-3[a,b,c]	2.1±0.4	9.0±0.4	1.2±0.1
PE	18:2, n-6[a,b,c]	4.8±0.2	6.5±0.3	8.8±0.6
	20:4, n-6[a,b,c]	30.8±0.9	9.0±0.3	22.0±1.1
	20:5, n-3[a]	0.1±0.0	1.3±0.4	-
	22:6, n-3[a,b,c]	12.1±0.9	33.2±0.7	6.3±0.9
PI	18:2, n-6[a,b]	2.6±0.5	5.9±0.3	6.1±0.3
	20:4, n-6[a,b,c]	29.8±1.01	20.1±0.7	24.2±1.3
	20:5, n-3[a]	0.1±0.1	1.0±0.1	-
	22:6, n-3[a,c]	0.1±0.1	2.7±0.3	0.3±0.0
PS	18:2, n-6[a,b]	2.3±0.2	3.4±0.4	3.1±0.3
	20:4, n-6[a,b,c]	8.0±0.3	2.6±0.8	5.2±0.5
	20:5, n-3	0.2±0.2	0.1±0.0	-
	22:6, n-3[a,c]	12.4±1.5	10.8±1.5	4.2±0.7

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between individual fatty acid species within each phospholipid fraction. [a] control *vs* n-3 PUFA, [b] control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA.

The n-6 PUFA diet increased the proportion of 18:2, n-6 in all fractions compared to control, approximately doubling it in PE and PI. However, in all fractions bar PC,

there was a paradoxical decrease in 20:4, n-6; of the order of 20-25% in PI and PE. Of the n-3 fatty acids, 20:5, n-3 was not detected in relation to the n-6 PUFA diet and 22:6, n-3 was less than half that of control except in PI where it was slightly higher.

A noticeable effect of the n-3 PUFA diet was the fall in the n-6:n-3 ratios which reached 8-fold in PI (Table 5.13). Significant changes in the double bond index, however, were restricted to PC and PE, where this parameter rose. In relation to the n-6 PUFA diet, the n-6:n-3 ratios rose 2 to 3-fold but the double bond index fell significantly in PC, PE and PS.

TABLE 5.13 *Phospholipid fraction n-6:n-3 ratios and double bond indices (DBI) in hearts isolated from rats fed experimental diets.*

Phospholipid fraction,		Diet		
n-6:n-3 ratio and DBI		Control	n-3 PUFA	n-6 PUFA
PC	n-6:n-3 ratio[a,b,c]	14.0±1.1	2.1±0.1	26.6±1.8
	DBI[a,b]	154±3	174±3	150±4
PE	n-6:n-3 ratio[a,b,c]	2.7±0.3	0.4±0.0	5.6±0.4
	DBI[a,b,c]	245±4	271±6	210±7
PI	n-6:n-3 ratio[a,b,c]	37.3±4.3	4.7±0.3	64.0±8.1
	DBI	154±9	164±6	128±6
PS	n-6:n-3 ratio[a,b,c]	1.7±0.3	0.7±0.1	5.2±1.0
	DBI[b]	174±14	169±10	144±8

Values: mean ± s.e.m., n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between parameters for individual phospholipid fractions. [a] control *vs* n-3 PUFA, [b] control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA.

5.3.8 Correlations: LV responses and cardiac fatty acid composition

In each dietary group, it was possible to identify and select the responses of hearts for which fractionated phospholipid fatty acid data were available (n=8 per group). Thus, baseline and maximum peak LVP could be regressed against the values for individual

fatty acid species, n-6:n-3 ratios and double bond indices, singly and in multiple combinations, for each phospholipid fraction (see section 2.9). No significant correlations were found.

5.4 Results: chemical denervation study

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5.4.1 Dietary PUFA and LVP: denervated hearts

In contrast to the findings in the absence of chemical denervation (see Table 5.2), baseline peak LVP was not influenced by diet in the denervated hearts (Table 5.14). The lack of difference was related to higher pressures in the n-3 PUFA dietary group.

TABLE 5.14 *Peak LVP (mm Hg) prior to noradrenaline infusion, in the presence of timolol 10 μM, in hearts isolated from chemically denervated rats fed experimental diets.*

Diet	LVP
n-3 PUFA	80±5
n-6 PUFA	84±5

Values: mean ± s.e.m, n=15 per dietary group.

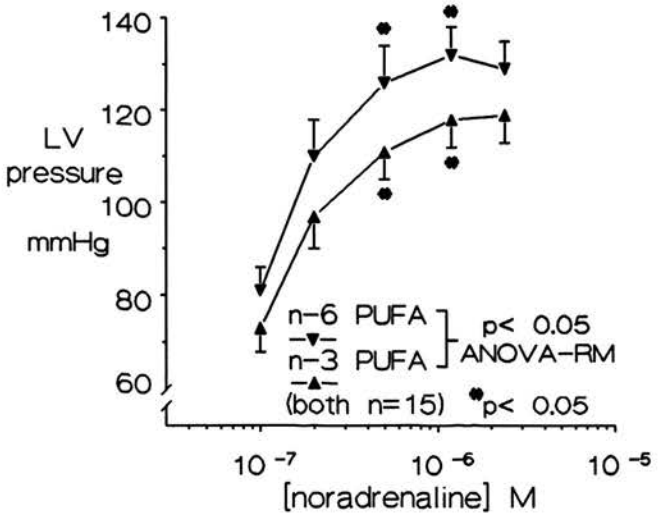

FIGURE 5.4 *Response of peak LVP (mm Hg) to noradrenaline (0.1-2.4 μm), in the presence of timolol (10 μM), of hearts isolated from chemically denervated rats fed experimental diets: n-3 PUFA and n-6 PUFA.*

In both dietary groups, peak LVP fell slightly from baseline with the first dose of noradrenaline (n-3 PUFA: 80±5 to 73±6 mm Hg, n-6 PUFA: 84±5 to 81±5 mm Hg, both N.S.). Overall, hearts from the n-3 PUFA group showed attenuated responses to α_1-adrenoceptor stimulation ($p< 0.05$, ANOVA-RM, Fig. 5.4). Secondary analysis identified significant differences at higher concentrations of agonist ($p< 0.05$, *t* test).

5.4.2 Dietary PUFA and LVdP.dt^{-1}: denervated hearts

LVdP.dt^{-1} was similar in the two dietary groups. In response to α_1-adrenoceptor stimulation, dP.dt$^{-1}_{max}$ and dP.dt$^{-1}_{min}$ both increased significantly ($p< 0.001$, ANOVA) but neither was altered in relation to diet (ANOVA-RM, N.S., Table 5.15).

TABLE 5.15 *LVdP.dt^{-1} (mm Hg.sec^{-1}) at baseline and in response to noradrenaline (0.1-2.4 μM) in the presence of timolol (10 μM) in hearts isolated from chemically denervated rats fed experimental diets.*

Noradrenaline concentration	Diet			
	n-3 PUFA		n-6 PUFA	
	dP.dt$^{-1}_{max}$	dP.dt$^{-1}_{min}$	dP.dt$^{-1}_{max}$	dP.dt$^{-1}_{min}$
Baseline	3473±102	2200±141	3627±122	2460±157
0.10 μM	3000±177	1893±174	3427±184	2400±167
0.19 μM	4053±176	2907±166	4160±101	3320±211
0.52 μM	4453±187	3373±179	4573±129	3813±210
1.22 μM	4533±153	3653±180	4733±153	4013±209
2.37 μM	4467±149	3653±195	4667±160	3907±206

Values: mean ± s.e.m., n=15 per dietary group. There were no significant differences in relation to diet.

5.4.3 Dietary PUFA and heart rate: denervated hearts

At baseline, hearts from the n-6 PUFA group had a faster heart rate but not significantly so (Table 5.16). There was again a trend to initial bradycardia but this was also not significant. Heart rates during α_1-adrenoceptor stimulation tended to be lower

in relation to the n-3 PUFA diet but there was no statistically significant difference between the groups (ANOVA-RM, N.S.).

TABLE 5.16 *Rates (bpm) at rest and in response to noradrenaline (0.1-2.4 μM) in the presence of timolol (10 μM) of hearts isolated from chemically denervated rats fed experimental diets.*

Noradrenaline	Diet	
concentration	n-3 PUFA	n-6 PUFA
Baseline	252±9	269±9
0.10 μM	232±8	254±9
0.19 μM	225±5	241±7
0.52 μM	234±4	250±7
1.22 μM	244±6	254±6
2.37 μM	252±5	266±6

Values: mean ± s.e.m., n=15 per dietary group. There were no significant differences between the groups.

5 4.4 Dietary PUFA: coronary flow and lactate production: denervated hearts

The hearts of animals fed the n-3 and n-6 PUFA diets did not differ in terms of coronary flow or lactate production (Table 5.17).

TABLE 5.17 *Resting coronary flow ($ml.min^{-1}.g$ wet heart $weight^{-1}$) and lactate production ($\mu mol.min^{-1}.g$ wet heart $weight^{-1}$) of hearts isolated from chemically denervated rats fed experimental diets.*

Diet	Flow	Lactate
n-3 PUFA	11.2±0.36	0.62±0.4
n-6 PUFA	12.3±0.48	0.67±0.4

Values: mean ± s.e.m., n=15 per dietary group. There were no significant differences between the groups.

5.4.5 Noradrenaline content: denervated hearts

Cardiac noradrenaline content did not differ in relation to the n-3 PUFA and n-6 PUFA diets (Table 5.18). In relation to 6-hydroxy-dopamine treatment, cardiac noradrenaline was significantly reduced (to $\leq 0.6\%$) regardless of diet.

TABLE 5.18 *Cardiac noradrenaline content (nmol.g wet heart weight^{-1}) in hearts isolated from rats fed experimental diets: effect of denervation with 6-hydroxy-dopamine.*

Diet	Normal	Denervated
n-3 PUFA	7.61±1.43[a]	0.04±0.03[a]
n-6 PUFA	7.56±1.28[b]	0.05±0.04[b]

Values: mean ± s.e.m., n=8 per dietary group. [a,b] $p < 0.001$
There were no significant differences in relation to diet.

5.4.6 Body and heart weights: denervated animals.

In this experiment, both the final body weights and the wet heart weights were significantly greater for rats fed the n-3 PUFA diet. The initial body weights were identical (Table 5.19).

TABLE 5.19 *Final body weights (g) and wet heart weights (g) of chemically denervated rats fed experimental diets.*

Diet	Initial body weight	Final body weight	Heart weight
n-3 PUFA	163±3	383±8[a]	1.2±0.0[b]
n-6 PUFA	163±3	347±8[a]	1.0±0.0[b]

Values: mean ± s.e.m., n=15 per dietary group. [a,b] $p < 0.05$.

5.4.7 Fatty acid composition of adipose tissue: denervated animals

In adipose tissue, the proportion of 18:2, n-6 varied 2-fold with respect to diet but 20:4, n-6 was not altered (Table 5.20). Fatty acids of the n-3 series were not detected ($< 0.1\%$) in relation to the n-6 PUFA diet.

TABLE 5.20 *Fatty acid composition of adipose tissue from chemically denervated rats fed experimental diets.*

	Diet	
Fatty acid species	n-3 PUFA	n-6 PUFA
18:2, n-6	14.2±0.7[a]	25.9±1.5[a]
20:4, n-6	0.4±0.0	0.4±0.0
20:5, n-3	1.3±0.1	-
22:6, n-3	1.9±0.2	-

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. [a] $p < 0.0001$.

5.4.8 Fatty acid composition of cardiac total and fractionated phospholipid

The amount of total phospholipid and the distribution of the individual phospholipid fractions was not affected by diet (Table 5.21).

TABLE 5.21 *Total phospholipid and phospholipid fractions in hearts isolated from chemically denervated rats fed experimental diets.*

Phospholipid	Diet	
fraction	n-3 PUFA	n-6 PUFA
PC	4140±233	4339±175
PE	3550±148	3351±168
PI	443±14	439±12
PS	244±13	248±12
Total	11052±224	11327±212

Values (mean ± s.e.m.) are expressed as μg fatty acids.g wet heart weight^{-1}, n=8 per dietary group. There were no significant differences between the groups.

The analysis of total and fractionated phospholipid fatty acid composition revealed many statistically significant differences in relation to diet. In total phospholipid, the proportion of 18:2, n-6 was the same with respect to the two PUFA diets but there was a 2-fold differences in the level of 20:4, n-6 (Table 5.22). Of the n-3 series, 20:5, n-3 was not detected (< 0.1%) in relation to the n-6 PUFA diet which was associated with a total phospholipid 22:6, n-3 content 30% of that brought about by the n-3 PUFA diet. There was a 6-fold difference in the n-6:n-3 ratios and the double bond index was significantly greater for the n-3 PUFA group.

TABLE 5.22 *Fatty acid composition, including the n-6:n-3 ratio and double bond index (DBI), of total phospholipid in hearts isolated from chemically denervated rats fed experimental diets.*

Fatty acid species,	Diet	
n-6:n-3 ratio and DBI	n-3 PUFA	n-6 PUFA
18:2, n-6	24.7±0.6	25.4±0.5
20:4, n-6	11.0±0.2[a]	20.1±0.1[a]
20:5, n-3	1.7±0.1	-
22:6, n-3	18.4±0.4[b]	5.1±0.2[b]
n-6:n-3 ratio	1.6±0.1[c]	9.2±0.5[c]
DBI	234±3[d]	210±1[d]

Values: mean ± s.e.m., n=8 per dietary group. Data for fatty acids represent the percentage weight of total fatty acid species. Superscript characters denote significant differences ($p < 0.001$) between individual fatty acid species and parameters.

The fatty acid composition of fractionated phospholipid revealed that 18:2, n-6 was unaltered with respect to diet, apart from in PE where it was slightly increased by n-6 PUFA (Table 5.23). The proportion of 20:4, n-6 varied up to 2-fold in PC, PE and PS but the difference was smaller in PI. Again, 20:5, n-3 was only detected in relation to

the n-3 PUFA diet which brought about 3 to 6 times greater levels of 22:6, n-3 than the n-6 PUFA diet.

TABLE 5.23 *Fatty acid composition of phospholipid fractions in hearts isolated from chemically denervated rats fed experimental diets.*

Phospholipid fraction and fatty acid species		Diet	
		n-3 PUFA	n-6 PUFA
PC	18:2, n-6	16.0±0.8	15.2±0.5
	20:4, n-6	14.0±0.4[a]	22.9±0.6[a]
	20:5, n-3	2.0±0.1	-
	22:6, n-3	9.0±0.7[b]	1.5±0.2[b]
PE	18:2, n-6	7.2±0.3[c]	9.0±0.3[c]
	20:4, n-6	9.9±0.2[d]	21.3±0.6[d]
	20:5, n-3	2.1±0.1	-
	22:6, n-3	32.0±1.0[e]	9.9±0.4[e]
PI	18:2, n-6	7.0±0.1	6.8±0.3
	20:4, n-6	24.8±0.9[g]	27.8±0.9[g]
	20:5, n-3	1.0±0.2	-
	22:6, n-3	2.9±0.3[h]	0.4±0.1[h]
PS	18:2, n-6	2.9±0.2	2.8±0.2
	20:4, n-6	2.5±0.2[i]	5.2±0.4[i]
	20:5, n-3	0.1±0.1	-
	22:6, n-3	19.4±1.8[j]	6.1±1.0[j]

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. Superscript characters denote significant differences ($0.0001 < p < 0.05$) between individual fatty acid species within fractions in relation to diet.

There were striking variations in the n-6:n-3 ratio, ranging from 8-fold in PE to 11-fold in PI (Table 5.24). The double bond indices were higher in all fractions in relation to the n-3 PUFA diet, though not significantly so in PI.

TABLE 5.24 *Phospholipid fraction n-6:n-3 ratios and double bond indices in hearts isolated from chemically denervated rats fed experimental diets.*

Phospholipid fraction,		Diet	
n-6:n-3 ratio and DBI		n-3 PUFA	n-6 PUFA
PC	n-6:n-3 ratio	2.3±0.1[a]	23.8±3.3[a]
	DBI	178±5[b]	162±4[b]
PE	n-6:n-3 ratio	0.5±0.0[c]	4.1±0.2[c]
	DBI	282±5[d]	238±3[d]
PI	n-6:n-3 ratio	6.0±0.5[e]	67.0±7.6[e]
	DBI	159±5	146±5
PS	n-6:n-3 ratio	0.4±0.0[f]	4.0±0.4[f]
	DBI	172±9[g]	142±10[g]

Values: mean ± s.e.m., n=8 per dietary group. Superscript characters denote significant differences ($0.0001 < p < 0.05$) between parameters for individual phospholipid fractions.

5.4.9 Changes in phospholipid fatty acid composition in relation to denervation

We compared the effects of the PUFA diets on cardiac fractionated phospholipid fatty acid composition in the first study with the effects in the second (denervation) study. Analysis of variance (section 2.8) confirmed that there was no significant variation in the fatty acid composition of PC and PI between the studies. Variance in PE arose from the smaller fall in 22:6, n-3 in the second study in relation to the n-6 PUFA diet (9.9±0.4 *vs* 6.3±0.9). However, these differences were not of great magnitude. More interesting was variation in the effects of the n-3 PUFA diet in PS whereby the proportion of 22:6, n-3 in the second study was considerably greater than in the first (19.4±1.8 *vs* 10.8±1.5). The remaining fatty acid species in PE and PS did not vary.

There was no significant variation in the n-6:n-3 ratios between the two studies and the double bond index only varied in PI, where it was greater in relation to n-6 PUFA following denervation (146±5 *vs* 128±6).

5.4.10 Correlation of LV responses with cardiac fatty acid composition

As in the preceding experiment, no correlation could be identified between the LV responses and the values for individual fatty acid species, n-6:n-3 ratios and double bond indices, singly or in multiple combinations, in the different phospholipid fractions.

5.5 Discussion

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The first of these two studies demonstrated that a 10% fat calorie diet rich in n-3 PUFA (30% of fat calories), compared to a relatively saturated control diet (10% fat calories, P/S ratio 0.3), was associated with the attenuation of peak LVP responses to noradrenaline in the isolated retrogradely perfused rat heart. The responses were obtained in the presence of timolol and were previously shown to be abolished by prazosin in this model, thus we infer that they were mediated by the α_1-adrenoceptor. A 10% fat calorie diet rich in n-6 PUFA (~50% of fat calories, almost all 18:2, n-6) had no statistically significant effect, but peak LVP responses were slightly enhanced compared to control. Comparison of peak LVP for the two PUFA diets confirmed that they differed significantly. There were small increases in $LVdP.dt^{-1}$ in response to α_1-adrenoceptor stimulation but these were not influenced by diet. Neither heart rate nor baseline coronary flow was influenced by diet. Compared to the control diet, both PUFA diets brought about widespread statistically significant changes in phospholipid fatty acid composition.

Subsequently, the n-3 and n-6 PUFA diets, which had differed the most in terms of LVP responses in the first study, were compared again in the context of chemical denervation. The difference in baseline peak LVP between the n-3 and n-6 PUFA diets disappeared when animals were denervated, but the separation of responses to noradrenaline was maintained. This suggests that, in this experimental model, dietary

n-3 PUFA may normally be associated with a reduction in spontaneous noradrenaline release. As in the first study, $LVdP.dt^{-1}$, heart rate and coronary flow did not differ between the two PUFA groups. Following chemical denervation, the distribution of individual fatty acid species within the phospholipid fractions for the n-3 and n-6 PUFA diets was very similar to that of the previous study with the exception of the 22:6, n-3 component in PS. We are not aware of any evidence to suggest that the metabolism of PS in relation to the sympathetic nervous system is distinct from that of the other three phospholipid fractions analysed.

Although we agree with the contention of Reibel *et al.* [1988] that dietary fish oil can reduce α_1-adrenoceptor mediated cardiac inotropy, we disagree with respect to the parameter(s) affected. These authors reported that fish oil decreased increments in $LVdP.dt^{-1}_{max}$, (to phenylephrine in the presence of propranolol), but we could not demonstrate any change in either $LVdP.dt^{-1}_{max}$ or $LVdP.dt^{-1}_{min}$ with respect to diet. The changes we observed were confined to peak LVP, a parameter not reported in this other study. The attenuation of peak LVP by dietary n-3 PUFA in our study is consistent with the decreases in resting tension, and tension responses to Ca^{2+}, described for papillary muscles isolated from rats fed an n-3 PUFA diet [McLennan *et al.*, 1987]. These findings, and ours, contrast with the lack of effects of n-3 PUFA in swine, where cardiac parameters at rest and during rapid atrial pacing were unaltered in animals fed mackerel oil as opposed to saturated fat [Hartog *et al.*, 1987*a*, Hartog *et al.*, 1987*b*].

There was no significant effect of the 10% fat calorie n-6 PUFA diet. Dietary n-6 PUFA in 30-35% fat calorie diets has been reported to improve the relationship between LV work and filling pressure [de Deckere and ten Hoor, 1980] and to enhance LV contractile force [Hoffman *et al.*, 1982] in the isolated rat heart. (Increased baseline coronary flow with n-6 PUFA was also reported in these studies but the effect was not apparent in the working heart [de Deckere and ten Hoor, 1980] or at one year [Hoffman *et al.*, 1982].) Conversely, in the study referred to above [McLennan *et al.*, 1987], the tension responses of rat papillary muscles were reduced

following dietary n-6 PUFA. Adding to these inconsistencies, another investigation from this group, using a similar n-6 PUFA diet, found no effect on either resting or stimulated LV ejection fraction in marmosets [Charnock *et al.*, 1987]. Thus, the effects of different n-6 PUFA diets on cardiac parameters in different experimental models are not easy to interpret.

The mechanism underlying the effects of dietary n-3 PUFA in our two studies remains unclear. However, the changes in fractionated phospholipid fatty acid composition in relation to the n-3 PUFA diet suggest certain possibilities. The changes in the PI fraction were extensive; the proportion of 22:6, n-3 was up to 30-fold greater than with either of the other two diets and the proportion of 20:4, n-6 was substantially (50%) decreased compared to control. With a lower dose of n-3 PUFA in a 40% fat calorie diet we have previously found 20:4, n-6 to be tightly conserved in PI (Chapters 3 and 4). If we can assume that the fatty acid composition of PI reflects that of PIP_2, the source of the second messengers inositol-*tris*-phosphate and diacylglycerol, then α_1-adrenoceptor signalling may have been affected in terms of second messenger composition and release. This is unlikely to be the sole explanation for the effects of n-3 PUFA as no correlation could be found between the fatty acid composition of PI and the cardiac responses. The substantially lower levels of 20:4, n-6 and significantly higher double bond indices in PC and PE, as mentioned before, carry implications for prostanoid synthesis and cell membrane-linked events.

Enrichment of the diet with n-6 PUFA had no significant effect on peak LVP responses compared to control despite 18:2, n-6 accounting for almost all the dietary fat calories. The explanation for the lack of effect may lie in the relatively limited changes the n-6 PUFA diet produced in fractionated phospholipid. Levels of 18:2, n-6 altered little; differences in 18:2, n-6 between the two PUFA diets were only seen in PE, and 20:4, n-6 was either unchanged or decreased compared to the control diet. Interestingly, the double bond index always fell in relation to dietary n-6 PUFA.

EDRF is released from coronary arteries [Collins *et al.*, 1986, Nyborg, 1990, Lamontagne *et al.*, 1992] and the endocardium [Shah *et al.*, 1991] and can reduce

myocardial contractility. Enhancement of the actions of EDRF by n-3 PUFA might be relevant to our observations and this is discussed in detail in Chapter 9.

In summary, a high dose of dietary n-3 PUFA (3.0% dietary calories in a 10% fat calorie diet) was associated with the attenuation of peak LVP responses to α_1-adrenoceptor stimulation in the isolated rat heart. Changes in fractionated phospholipid fatty acid composition suggested certain possible mechanisms in the n-3 PUFA effect. The amount of n-3 PUFA administered was very large indeed and it remains to be seen whether a realistic dose might have similar consequences. In the following chapter, the effects of dietary PUFA on the heart are pursued in the context of semisynthetic diets relevant to man.

Chapter 6

Polyunsaturated fatty acids in 40% fat calorie diets and the heart

6.1 Introduction

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In Chapter 5 we demonstrated that the responses of peak LVP to α_1-adrenoceptor stimulation in the isolated rat heart could be attenuated by dietary n-3 PUFA compared to a control relatively saturated diet. Dietary n-6 PUFA did not exert this effect. For these studies we used diets deriving 10% of dietary calories from fat in an attempt to reproduce published findings [Reibel *et al.*, 1988] without the shortcomings of their design. Such diets are of limited relevance to man. The two PUFA diets are too rich in their respective PUFA series and all three diets are too low in fat. Having identified responses which were susceptible to dietary influence in our experimental model, we wished to pursue the possible effects of the diets described in Chapters 3 and 4; diets which represent what is, or might be, consumed by man. Accordingly, we examined the effects of dietary PUFA in 40% fat calorie diets on the responses to α_1-adrenoceptor stimulation in the isolated rat heart. As before, the control diet had a P/S ratio of 0.3 with an adequate n-6 essential fatty acid intake. Supplementation of the control diet with 0.4% dietary calories fish oil provided the n-3 PUFA diet and the n-6 PUFA diet had a P/S ratio of 2.0.

6.2 Methods

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Thirty-six Sprague-Dawley rats were separated at random into three groups and commenced on semisynthetic experimental diets deriving 40% of total dietary calories from fat. Briefly, 24 animals received a control diet of P/S ratio 0.3, and 12 of these received a daily oral supplement of n-3 PUFA (0.4% total dietary calories). A third group of 12 animals received an n-6 PUFA diet of P/S ratio 2.0. The control and n-6 PUFA groups received a daily oral dose of olive oil which was retained from their daily dietary fat intake (see section 2.2.4). At the end of the eight week feeding period, hearts were perfused (section 2.6.3) and the responses of the isolated hearts to noradrenaline 0.1-2.4 μM in the presence of timolol 10 μM were recorded according to the protocol described in section 2.6.4. No experimental exclusions were indicated.

6.3 Results

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6.3.1 Dietary PUFA and LVP

At baseline, in the absence of noradrenaline but in the presence of timolol, peak LVP was lower for the two PUFA groups but neither differed significantly from control (Table 6.1).

TABLE 6.1 *Peak LVP (mm Hg) prior to noradrenaline infusion, in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets.*

Diet	LVP
Control	81±6
n-3 PUFA	72±4
n-6 PUFA	78±4

Values: mean ± s.e.m., n=12 per dietary group. There were no significant differences between the groups.

The response of peak LVP to α_1-adrenoceptor stimulation in hearts from the n-3 PUFA group tended to be less than that of the control group but the difference was not significant (ANOVA-RM, Fig. 6.1).

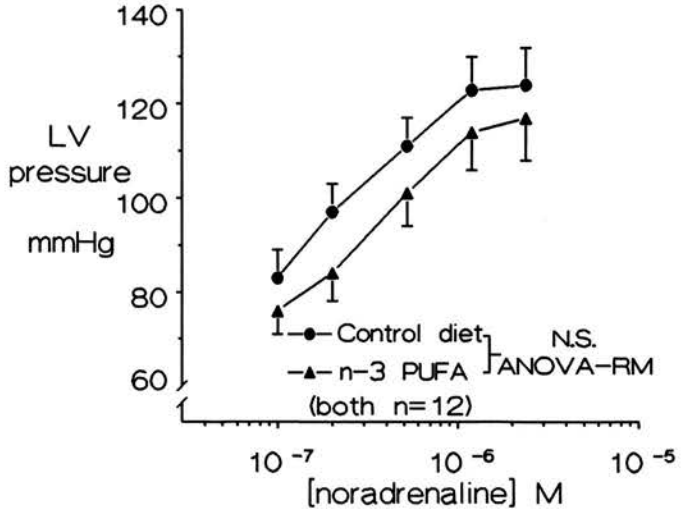

FIGURE 6.1 *Response of peak LVP (mm Hg) to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets: n-3 PUFA.*

In contrast, the responses for the n-6 PUFA dietary group were significantly attenuated in a non-parallel manner compared to control ($p< 0.05$, ANOVA-RM, Fig. 6.2).

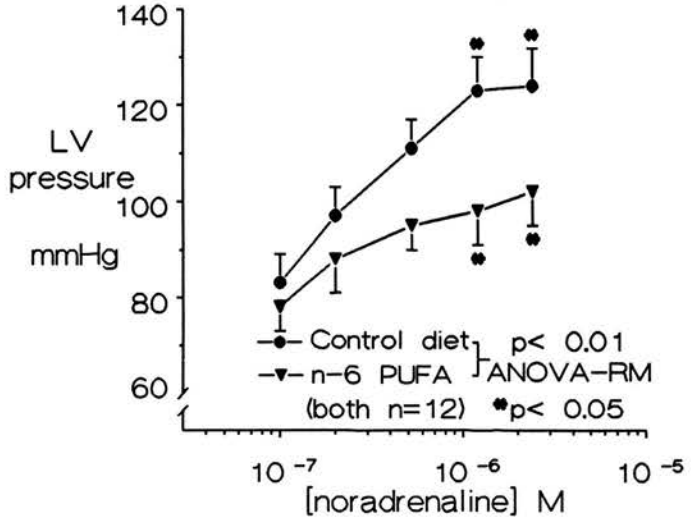

FIGURE 6.2 *Response of peak LVP (mm Hg) to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets: n-6 PUFA.*

Secondary analysis revealed significant differences between the n-6 PUFA and control groups at higher concentrations of noradrenaline. The responses for the n-6 PUFA group tended to diverge from those of the n-3 group ($p = 0.06$, ANOVA-RM).

6.3.2 Dietary PUFA and LVdP.dt^{-1}

Neither $dP.dt^{-1}_{max}$ nor $dP.dt^{-1}_{min}$ were altered at baseline with respect to diet. Both parameters increased significantly ($p < 0.001$, ANOVA) in response to α_1-adrenoceptor stimulation but there were no significant differences (ANOVA-RM) between the three dietary groups (Tables 6.2 and 6.3).

TABLE 6.2 *$LVdP.dt^{-1}_{max}$ (mm Hg.sec^{-1}) at baseline and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets.*

Noradrenaline	Diet		
concentration	Control	n-3 PUFA	n-6 PUFA
Baseline	3008±250	2850±121	3083±149
0.10 μM	3083±249	3000±118	3200±232
0.19 μM	3583±278	3417±158	3833±280
0.52 μM	3983±237	3817±215	4150±209
1.22 μM	4300±252	4267±236	4217±210
2.37 μM	4333±280	4300±303	4083±236

Values: mean ± s.e.m., n=12 per dietary group. There were no significant differences in relation to diet.

TABLE 6.3 *$LVdP.dt^{-1}_{min}$ (mm Hg.sec^{-1}) at baseline and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets.*

Noradrenaline		Diet	
concentration	Control	n-3 PUFA	n-6 PUFA
Baseline	2167±153	2050±100	2250±128
0.10 μM	2250±167	2100±101	2258±152
0.19 μM	2717±194	2383±134	2683±202
0.52 μM	3283±215	2983±127	3133±144
1.22 μM	3650±222	3333±164	3283±158
2.37 μM	3567±248	3233±218	3250±197

Values: mean ± s.e.m., n=12 per dietary group. There were no significant differences in relation to diet.

6.3.3 Dietary PUFA and heart rate

TABLE 6.4 *Heart rate (bpm) at baseline and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from rats fed experimental diets.*

Noradrenaline		Diet	
concentration	Control	n-3 PUFA	n-6 PUFA
Baseline	309±8[a]	316±8	331±6[a]
0.10 μM	296±8	304±8	312±7
0.19 μM	287±8	293±8	291±7
0.52 μM	286±6	292±8	292±9
1.22 μM	286±7	292±6	293±9
2.37 μM	293±8	296±7	303±9

Values: mean ± s.e.m., n=12 per dietary group. [a] $p < 0.05$.

At baseline, the heart rate of the n-6 PUFA group was significantly greater than that of the control group, but not than that of the n-3 PUFA group. This difference was no longer apparent at the lowest concentration of agonist, and the dose response relationships for the three groups did not vary with respect to diet (Table 6.4). Heart rates were generally slower, but did not alter significantly, during α_1-adrenoceptor stimulation.

6.3.4 Dietary PUFA: coronary flow and lactate production

In this study, coronary flow was assessed at baseline, twice during α_1-adrenoceptor stimulation (at noradrenaline concentrations 0.19 μM and 2.37 μM) and on recovery 3 minutes post-noradrenaline infusion (Table 6.5). At none of these points did the three dietary groups differ significantly from one another. Coronary flow did decrease in all three groups with increasing concentrations of noradrenaline.

TABLE 6.5 *Coronary flow rates ($ml.min^{-1}.g$ wet heart $weight^{-1}$) of hearts isolated from rats fed experimental diets.*

	Diet		
Coronary flow	Control	n-3 PUFA	n-6 PUFA
Baseline	10.6±0.5	11.8±0.6	11.4±0.4
0.19 μM	9.2±0.3	8.7±0.4	7.9±0.6
2.37 μM	8.4±0.6	8.1±0.4	7.7±0.5
Recovery†	9.6±0.5	9.5±0.4	9.1±0.8

Values: mean ± s.e.m., n=12 per dietary group. There were no significant differences in relation to diet. †3 minutes post-noradrenaline infusion.

Baseline lactate production was not influenced by diet (Table 6.6).

TABLE 6.6 *Production of lactate ($\mu mol.min^{-1}.g$ wet heart $weight^{-1}$) by hearts isolated from rats fed experimental diets.*

Diet	Lactate
Control	0.50±0.04
n-3 PUFA	0.57±0.02
n-6 PUFA	0.56±0.04

Values: mean ± s.e.m., n=12 per dietary group. There were no significant differences between the groups.

6.3.5 Dietary PUFA: body and heart weights

Rats fed the n-3 PUFA diet were heavier, and those fed the n-6 PUFA diet lighter, than control, but neither significantly so (Table 6.7).

TABLE 6.7 *Final body weights (g) and heart weights (g) of rats fed experimental diets.*

Diet	Body weight	Heart weight
Control	332±13	1.00±0.04
n-3 PUFA	352±14	1.08±0.03
n-6 PUFA	318±8	0.97±0.02

Values: mean ± s.e.m., n=12 per dietary group. There were no significant differences between the groups.

6.3.6 Fatty acid composition of adipose tissue

The proportion of 18:2, n-6 did not alter in relation to n-3 PUFA supplementation but the small percentage of adipose 20:4, n-6 was decreased (Table 6.8). Only in the n-3 PUFA group was 22:6, n-3 detectable in adipose tissue; 20:5, n-3 was not apparent for any of the diets. The P/S ratio 2.0 n-6 PUFA diet more than doubled the proportion of 18:2, n-6 and was associated with a small but significant increase in 20:4, n-6.

TABLE 6.8 *Fatty acid composition of adipose tissue from rats fed experimental diets.*

	Diet		
Fatty acid species	Control	n-3 PUFA	n-6 PUFA
18:2, n-6	13.7±0.2[a]	13.4±0.1[b]	31.3±0.3[a,b]
20:4, n-6	0.2±0.0[c,d]	0.1±0.0[c,e]	0.3±0.0[d,e]
20:5, n-3	-	-	-
22:6, n-3	-	0.1±0.0	-

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. Superscript characters denote significant differences ($0.05 < p < 0.001$) between individual fatty acid species in relation to diet.

6.3.7 Fatty acid composition of cardiac total and fractionated phospholipid

The distribution of the individual phospholipid fractions and the amount of total phospholipid were unaffected by diet (Table 6.9).

TABLE 6.9 *Total phospholipid and phospholipid fractions in hearts isolated from rats fed experimental diets.*

Phospholipid		Diet	
fraction	Control	n-3 PUFA	n-6 PUFA
PC	5081±161	4832±158	5236±250
PE	3287±200	3430±123	3233±228
PI	470±11	484±12	474±13
PS	265±22	266±19	277±21
Total	11233±272	11611±275	11531±256

Values (mean ± s.e.m.) are expressed as μg fatty acids.g wet heart weight^{-1}, n=8 per dietary group. There were no significant differences between the groups.

The fatty acid composition of both total and fractionated phospholipid revealed a number of statistically significant changes. In total phospholipid (Table 6.10), the n-3 PUFA supplement did not alter 18:2, n-6 compared to the control diet but there was a 25% fall in 20:4, n-6. Only in relation to dietary n-3 PUFA was 20:5, n-3 detected and 22:6, n-3 was nearly doubled. The n-6:n-3 ratio was more than halved and the double bond index increased significantly. With the P/S ratio 2.0 n-6 PUFA diet, there was a small increase in 18:2, n-6 but 20:4, n-6 did not change. The proportion of 22:6, n-3 fell slightly with dietary n-6 PUFA and there was a 50% rise in the n-6:n-3 ratio but the double bond index did not differ significantly from control.

TABLE 6.10 *Fatty acid composition of total phospholipid, including the n-6:n-3 ratio and double bond index (DBI), in hearts isolated from rats fed experimental diets.*

Fatty acid species.	Diet		
n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
18:2, n-6	19.6 ± 0.4^{a}	19.7 ± 0.4^{b}	$23.0\pm0.6^{a,b}$
20:4, n-6	24.0 ± 0.4^{c}	$18.7\pm0.5^{c,d}$	23.4 ± 0.3^{d}
20:5, n-3	-	0.3±0.0	-
22:6, n-3	9.5±0.3	17.2±0.6	7.5±0.3
n-6:n-3 ratio	4.3 ± 0.1^{e}	$2.0\pm0.1^{e,f}$	6.1 ± 0.3^{f}
DBI	228 ± 2^{g}	$243\pm2^{g,h}$	223 ± 2^{h}

Values: mean ± s.e.m., n=8 per dietary group. Data for fatty acids represent the percentage weight of total fatty acid species. Superscript characters denote significant differences ($p< 0.0005$) between individual fatty acid species in relation to diet.

Examining the fatty acid composition of the phospholipid fractions (Table 6.11), supplementation of the diet with n-3 PUFA left 18:2, n-6 unaltered but 20:4, n-6 decreased in all fractions. The decrease was limited in PI and PS but substantial in PC and PE. With regard to the n-3 fatty acid species, 20:5, n-3 was only detected in PC and PE with n-3 PUFA supplementation and was not apparent in PI and PS with any

of the diets. The proportion of 22:6, n-3 rose more than 50% in PE and PS and more than doubled in PC and PI. In relation to the n-6 PUFA diet, there were modest increases in 18:2, n-6 in all fractions but 20:4, n-6 was unaltered compared to the control diet. As mentioned, 20:5, n-3 was not detected following the n-6 PUFA diet and 22:6, n-3 showed small decreases.

TABLE 6.11 *Fatty acid composition of phospholipid fractions in hearts isolated from rats fed experimental diets.*

Phospholipid fractions and fatty acid species		Diet		
		Control	n-3 PUFA	n-6 PUFA
PC	18:2, n-6[b,c]	8.3±0.5	9.1±0.6	11.5±0.7
	20:4, n-6[a,c]	29.3±0.6	23.8±0.7	29.4±0.6
	20:5, n-3	-	0.3±0.1	-
	22:6, n-3[a,b,c]	3.9±0.7	8.9±0.6	2.7±0.2
PE	18:2, n-6[b,c]	4.9±0.2	4.8±0.4	6.8±0.5
	20:4, n-6[a,c]	24.5±0.5	17.9±0.9	23.3±0.6
	20:5, n-3	-	0.3±0.1	-
	22:6, n-3[a,b,c]	18.9±0.5	29.8±2.2	16.6±0.9
PI	18:2, n-6[b,c]	4.2±0.2	4.1±0.2	6.9±0.2
	20:4, n-6[a]	36.9±0.6	34.9±0.5	35.7±0.4
	20:5, n-3	-	-	-
	22:6, n-3[a,b,c]	1.2±0.1	2.7±0.1	0.8±0.1
PS	18:2, n-6[c]	2.3±0.3	2.1±0.1	3.4±0.5
	20:4, n-6[a]	7.1±0.6	4.9±0.3	5.8±0.6
	20:5, n-3	-	-	-
	22:6, n-3[a,c]	12.9±1.3	20.6±1.6	10.7±0.8

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=8 per dietary group. Superscript characters denote significant differences ($0.0005 < p < 0.05$) between individual fatty acid species within phospholipid fractions. [a] control *vs* n-3 PUFA, [b] control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA

The n-6:n-3 ratios fell at least twofold with dietary n-3 PUFA which increased the double bond index significantly in PC and PE but not in PI and PS (Table 6.12) The most prominent effect of the P/S 2.0 n-6 PUFA diet was an increase in the n-6:n-3 ratio; up by a third in PC, PE and PS and by more than half in PI. However, the double bond index did not differ significantly from control following dietary n-6 PUFA.

TABLE 6.12 *Phospholipid fraction n-6:n-3 ratios and double bond indices (DBI) in hearts isolated from rats fed experimental diets.*

Phospholipid fraction,		Diet		
	n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
PC	n-6:n-3 ratio[a,b,c]	7.8±0.5	3.0±0.2	12.4±0.8
	DBI[a,c]	186±2	194±3	184±2
PE	n-6:n-3 ratio[a,b,c]	1.7±0.1	0.9±0.2	2.3±0.2
	DBI[a,c]	272±4	292±5	268±4
PI	n-6:n-3 ratio[a,b,c]	22.7±1.3	9.8±0.3	35.0±2.0
	DBI	183±3	183±3	179±2
PS	n-6:n-3 ratio[a,c]	1.6±0.2	0.5±0.0	2.0±0.1
	DBI	175±11	183±12	167±10

Values: mean ± s.e.m., n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between parameters for individual phospholipid fractions. [a] control *vs* n-3 PUFA, [b] control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA

6.3.8 Correlations: LV responses with fatty acid composition

The correlation of individual fatty acid species, n-6:n-3 ratios and double bond indices, singly and in multiple combinations, in each phospholipid fraction with the changes in LVP failed to reveal any significant relationships.

6.4 Discussion

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This study demonstrated once more that the responses of the isolated rat heart to α_1-adrenoceptor stimulation can be influenced by dietary PUFA. However, the findings are in marked contrast to those of Chapter 5 where a 10% fat diet rich in n-3 PUFA, but not a diet rich in n-6 PUFA, significantly attenuated peak LVP responses to noradrenaline in the presence of timolol. With the 40% fat diets, supplementation of a relatively saturated (P/S ratio 0.3) control diet with a small amount of n-3 PUFA (0.4% dietary calories) tended to reduce peak LVP responses but the effect was not significant. Conversely, an n-6 PUFA diet (P/S ratio 2.0) did significantly attenuate peak LVP responses. $LVdP.dt^{-1}_{max}$, $LVdP.dt^{-1}_{min}$ and coronary flow were not affected by diet, either prior to or during α_1-adrenoceptor stimulation. Both 40% fat PUFA diets brought about numerous significant changes in phospholipid fatty acid composition.

With regard to the effects of dietary n-6 PUFA, the difference between the 10% and 40% studies is remarkable. Whereas the peak LVP responses of the 10% and 40% fat control groups are superimposable at 0.19-1.22 μM (see Figs. 5.2 and 6.2), the significant effect of n-6 PUFA in the current study is opposite in direction to the previous trend. The reason for these contrasting results is unclear but there were several differences in the effects of the two n-6 PUFA diets on phospholipid fatty acid composition. In total phospholipid, at 40% fat, 18:2, n-6 was higher in relation to the n-6 PUFA diet than control or n-3 PUFA diets (Table 6.10) but this was not the case at 10% fat where the n-3 and n-6 PUFA diets did not differ (Table 5.11). But it is the disparity in the effects of the two PUFA diets on 20:4, n-6 that is most striking. At 10% fat there was an unexpected decrease in 20:4, n-6 with n-6 PUFA whereas at 40% fat this fatty acid species remained unchanged by n-6 PUFA, as we commonly find. This observation extends to fractionated phospholipid fatty acid composition where paradoxical falls in 20:4, n-6 in PE, PI and PS were seen with the 10% fat n-6 PUFA diet (Table 5.12). This did not occur at 40% fat (Table 6.11).

In the present study, the small dose of n-3 PUFA (0.4% dietary calories) had no significant effect on the responses of the isolated heart in contrast to what was found with a higher dose (7.5% dietary calories) in a lower fat diet. The differences in tissue fatty acid composition in response to these two n-3 PUFA diets are extensive. In total and fractionated phospholipid (Tables 5.11, 5.12 and 6.10, 6.11) supplementation of the 40% fat diet with 0.4% total calories n-3 PUFA affected 20:4, n-6 to a lesser extent than was the case for the 10% fat n-3 PUFA diet. Higher levels of 22:6, n-3 for all three 40% fat diets meant that the relative increases in this fatty acid species brought about by the n-3 PUFA supplement were smaller. Interestingly, although the associated decreases in the n-6:n-3 ratio were curtailed, relative increases in the double bond index were similar (Tables 5.13 and 6.12). Comparison of the 10% and 40% fat diets is however complicated by the fact that the amount of fat, and not the composition, differed between the two control diets. As a result, there was less 22:6, n-3 in the cardiac phospholipids of rats fed the control 10% fat diet.

These differences between the effects on phospholipid fatty acid composition of the 10% and 40% fat diets may in some way account for the differences in their effects on peak LVP. However, the difficulty in interpreting these data is the lack of statistical support for a cause and effect relationship; we were unable to correlate fatty acid composition, n-6:n-3 ratio or double bond index with LVP. Indeed, this remained true when the data for the 10% fat and 40% fat experiments was pooled.

The effect of n-6 PUFA on the responses of the isolated heart in the present study contrasts again with reports of increased cardiac work and contractility following dietary n-6 PUFA [de Deckere and ten Hoor, 1980, Hoffman *et al.*, 1982]. Although peak LVP is not an index of contractility, we found no effect on $LVdP.dt^{-1}$. The findings of the present study are now consistent with the attenuation of rat papillary muscle tension responses in relation to an n-6 PUFA diet [McLennan *et al.*, 1987]. Once more, there was no effect of n-6 PUFA on coronary flow.

The mechanism underlying the effect of dietary n-6 PUFA in our study is unclear. Increases in phospholipid 18:2, n-6 following dietary n-6 PUFA did not lead to

increases in the prostanoid precursor 20:4, n-6 but a simple and attractive proposition is that myocardial prostanoid release was affected in our study. This possibility is examined in the following chapter.

In summary, a 40% fat calorie diet with an increased proportion of n-6 PUFA (P/S ratio 2.0), compared to a control relatively saturated diet (40% fat, P/S ratio 0.3), was associated with the attenuation of peak LVP responses to α_1-adrenoceptor stimulation in the isolated rat heart. A similar trend which was not significant was seen with a small dietary supplement of n-3 PUFA as might be taken by man. In Chapter 7, the influence of the cyclooxygenase inhibitor flurbiprofen on the effects of dietary PUFA is investigated.

Chapter 7

The rôle of prostanoids in the effect of dietary polyunsaturated fatty acids on the heart

7.1 Introduction

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In Chapter 6, we demonstrated that an n-6 PUFA diet (40% fat calories, P/S ratio 2.0) was associated with the attenuation of LVP responses to α_1-adrenoceptor stimulation in the isolated rat heart. A small dietary supplement of n-3 PUFA (0.4% dietary calories) produced a similar trend but this was not significant. Cardiac fractionated phospholipid fatty acid composition demonstrated many significant changes in relation to both of the PUFA diets but the mechanism underlying their effects on cardiac responses remains unclear. We thought it possible that increased release of series 2 prostanoids in relation to dietary n-6 PUFA might account for our findings.

The aim of the present study was to examine the influence of the cyclooxygenase inhibitor flurbiprofen on the effects of the n-6 PUFA diet. For two reasons, flurbiprofen was administered parenterally to the animals at the end of the dietary feeding period, as opposed to being infused during the experiments. First, we felt that it was of more interest to look at the effects of "chronic" cyclooxygenase inhibition in relation to the effects of diet on LV responses. Second, this strategy allowed us to assess any effect of the administration of flurbiprofen on phospholipid fatty acid composition.

7.2 Methods

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Forty-eight Sprague-Dawley rats were randomly separated into three groups of 16 and fed the 40% fat calorie semisynthetic experimental diets for eight weeks. The control relatively saturated diet (P/S ratio 0.3) was fed to 32 animals, 16 of which received a small daily oral supplement of fish oil (0.4% dietary calories) by gavage. The remaining 16 animals received an n-6 PUFA diet of P/S ratio 2.0. The control and n-6 PUFA groups received control oral doses of olive oil which was retained from their dietary fat intake (see section 2.2.4). From 48 hours prior to sacrifice, twelve rats from each group received flurbiprofen in a dose of 2 $mg.kg^{-1}$ intraperitoneally twice daily, such that each treated animal received five doses (total dose 10 $mg.kg^{-1}$). The four untreated rats in each group were controls for the effect of flurbiprofen. On the day of perfusion, hearts were excised (section 2.6.3) and perfused according to the protocol of section 2.6.4. The effect of flurbiprofen on cardiac prostanoid release was assessed by measuring coronary venous levels of 6-*oxo*-$PGF_{1\alpha}$, the breakdown product of prostacyclin (section 2.7.6). Timed samples of coronary effluent were collected for 6 hearts in each flurbiprofen-treated dietary group and for the 4 hearts from the control untreated animals in each dietary group. The samples were immediately frozen for subsequent assay. Subsequently, the responses of the hearts isolated from flurbiprofen-treated animals to noradrenaline 0.1-2.4 μM in the presence of 10 μM timolol were recorded. One heart from each group of 12 was excluded from further analysis.

7.3 Results

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7.3.1 Dietary PUFA and LVP

Baseline peak LVP, in the absence of noradrenaline but in the presence of timolol, did not vary with respect to diet (Table 7.1).

TABLE 7.1 *Baseline peak LVP (mm Hg), prior to noradrenaline infusion, in the presence of timolol 10 μM, of hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Diet	Pressure
Control	68±1
n-3 PUFA	69±1
n-6 PUFA	70±2

Values: mean ± s.e.m., n=11 per dietary group. There were no significant differences between the groups.

Despite a tendency to attenuation, the response of peak LVP to α_1-adrenoceptor stimulation in hearts from the n-3 PUFA group did not differ significantly from that of the control group (N.S., ANOVA-RM, Fig. 7.1).

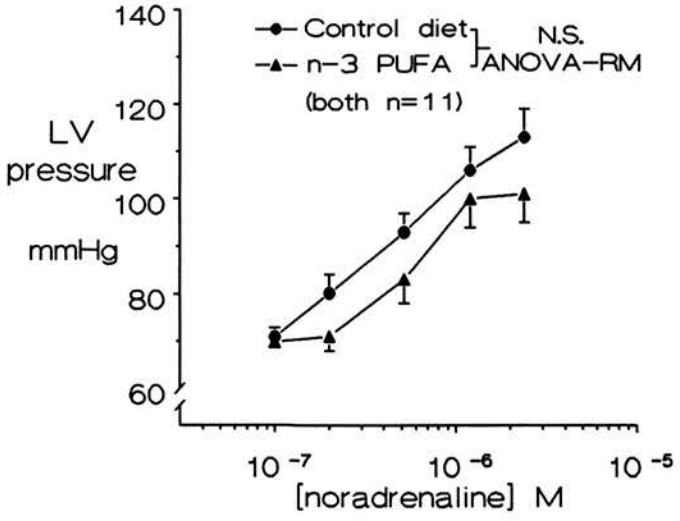

FIGURE 7.1 *Response of peak LVP (mm Hg) to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), of hearts isolated from flurbiprofen-treated rats fed experimental diets: n-3 PUFA.*

In contrast to the study of Chapter 6, the LVP responses of hearts from the n-6 PUFA group also did not differ significantly from control (N.S., ANOVA-RM), displaying only a slight tendency to attenuation at the highest doses of noradrenaline from control (Fig. 7.2).

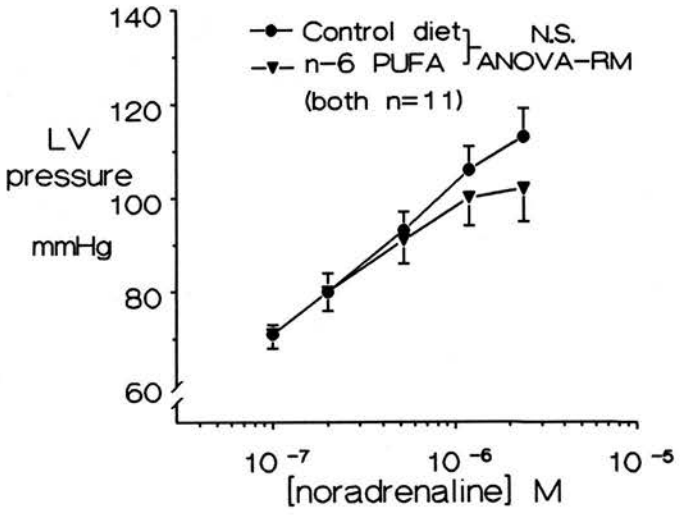

FIGURE 7.2 *Response of peak LVP (mm Hg) to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), of hearts isolated from flurbiprofen-treated rats fed experimental diets: n-6 PUFA.*

When the two PUFA groups were compared to one another, there was a non-significant tendency to attenuation in the n-3 PUFA group (p=0.07, ANOVA-RM).

7.3.2 Dietary PUFA and LVdP.dt^{-1}

Both LVdP.dt$^{-1}_{max}$ (Table 7.2) and LVdP.dt$^{-1}_{min}$ (Table 7.3) increased significantly in response to noradrenaline ($p < 0.001$, ANOVA), but neither varied with respect to diet (N.S., ANOVA-RM).

TABLE 7.2 *$LVdP.dt^{-1}_{max}$ (mm $Hg.sec^{-1}$) at baseline and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Noradrenaline concentration	Diet		
	Control	n-3 PUFA	n-6 PUFA
Baseline	2364±101	2409±1.3	2945±230
0.10 μM	2491±130	2509±101	3050±300
0.19 μM	3018±170	2645±140	3136±250
0.52 μM	3455±180	3164±190	3345±220
1.22 μM	3900±210	3764±210	3682±240
2.37 μM	4064±280	4000±260	3855±300

Values: mean ± s.e.m., n=11 per dietary group. There were no significant differences in relation to diet.

TABLE 7.3 *$LVdP.dt^{-1}_{min}$ (mm $Hg.sec^{-1}$) at baseline and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Noradrenaline concentration	Diet		
	Control	n-3 PUFA	n-6 PUFA
Baseline	1618±38	1700±49	1718±38
0.10 μM	1682±50	1691±55	1764±85
0.19 μM	2100±150	1845±110	2173±130
0.52 μM	2700±100	2400±170	2609±140
1.22 μM	3182±160	2955±160	2900±170
2.37 μM	3236±180	3109±170	2900±210

Values: mean ± s.e.m., n=11 per dietary group. There were no significant differences in relation to diet.

7.3.3 Dietary PUFA and heart rate

Baseline heart rates did not differ in relation to diet. With α_1-adrenoceptor stimulation, the heart rates for the n-3 PUFA group tended to be lower, and the heart rates for the n-6 PUFA group higher, than control (Table 7.4). Neither PUFA group differed from the control group. Comparison of the two PUFA groups confirmed that the heart rates were significantly higher in the n-6 PUFA group ($p< 0.05$, ANOVA-RM). Secondary analysis revealed significant differences between the two PUFA groups at the maximum doses of noradrenaline ($p< 0.05$, *t* test).

TABLE 7.4 *Heart rates (bpm) at baseline and in response to noradrenaline (0.1-2.4 μM), in the presence of timolol (10 μM), in hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Noradrenaline concentration	Diet		
	Control	n-3 PUFA	n-6 PUFA
Baseline	303±6	296±9	302±7
0.10 μM	294±5	282±8	294±5
0.19 μM	287±5	270±6	290±5
0.52 μM	289±5	279±6	296±5
1.22 μM	285±8	278±6[a]	300±4[a]
2.37 μM	284±9	279±6[b]	300±3[b]

Values: mean ± s.e.m., n=11 per dietary group. [a,b] $p< 0.05$

7.3.4 Dietary PUFA: coronary flow and lactate production

Baseline coronary flow and lactate production were comparable for the three dietary groups (Table 7.5).

TABLE 7.5 *Baseline coronary flow (ml.min^{-1}.g wet heart weight^{-1}) and lactate production (μmol.min^{-1}.g wet heart weight^{-1}) in hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Diet	Flow	Lactate
Control	10.9±0.4	0.13±0.08
n-3 PUFA	11.6±0.5	0.17±0.05
n-6 PUFA	11.3±0.2	0.17±0.06

Values: mean ± s.e.m., n=11 per dietary group. There were no significant differences between the groups.

7.3.5 Production of 6-oxo-PGF$_{1\alpha}$

Coronary venous levels of 6-oxo-PGF$_{1\alpha}$, whether following, or in the absence of, treatment with flurbiprofen, did not vary with respect to diet (Table 7.6). The flurbiprofen regimen led to significant decreases of approximately 75% in the production of 6-*oxo*-PGF$_{1\alpha}$.

TABLE 7.6 *Coronary venous 6-oxo-PGF$_{1\alpha}$ (ng.min^{-1}.g wet heart weight^{-1}) in hearts of rats fed experimental diets: effect of parenteral flurbiprofen (total 10 mg.kg^{-1}, i.p.).*

Diet	Untreated	Treated	Δ %
Control	3.73±0.93^{a}	0.92±0.17^{a}	-75.3
n-3 PUFA	3.95±0.71^{b}	0.87±0.22^{b}	-78.0
n-6 PUFA	3.80±0.30^{c}	0.97±0.15^{c}	-74.5

Values: mean ± s.e.m., n=4 (untreated) and n=6 (treated). Superscript characters denote significant differences $^{a\text{-}c}$ $p< 0.01$.

7.3.6 Body and heart weights

The final body weights of the n-3 PUFA group were significantly heavier than those of the n-6 PUFA group but neither differed from control; initial weights had not differed (Table 7.7). Heart weights were similar in the three groups.

TABLE 7.7 *Initial and final body weights (g) and heart weights (g) of flurbiprofen-treated rats fed experimental diets.*

Diet	Initial weight	Final weight	Heart weight
Control	130±2	384±4	1.17±0.02
n-3 PUFA	131±6	395±10[a]	1.18±0.02
n-6 PUFA	125±5	358±9[a]	1.15±0.03

Values: mean ± s.e.m., n=11 per dietary group. [a] $p < 0.05$.

7.3.7 Fatty acid composition of cardiac total and fractionated phospholipid

The amount of total phospholipid and the distribution of the individual fractions was unaffected by diet (Table 7.8).

TABLE 7.8 *Total phospholipid and phospholipid fractions in hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Phospholipid fractions	Diet		
	Control	n-3 PUFA	n-6 PUFA
PC	4237±320	4059±230	4295±330
PE	2890±269	2487±228	2829±299
PI	467±17	477±18	461±23
PS	202±14	218±13	229±19
Total	10906±329	10276±234	11289±451

Values (mean ± s.e.m.) are expressed as μg fatty acids.g wet weight^{-1}, n=8 per dietary group. There were no significant differences between the groups.

The fatty acid composition of total and fractionated phospholipid revealed numerous statistically significant changes with respect to diet. In total phospholipid, supplementation of the diet with n-3 PUFA did not affect 18:2, n-6 compared to control but 20:4, n-6 was significantly reduced (Table 7.9). Of the n-3 species, 20:5, n-3 was only detected following dietary n-3 PUFA which substantially increased the level of 22:6, n-3 and nearly halved the n-6:n-3 ratio and tended to increase the double bond index. In relation to dietary n-6 PUFA, 18:2, n-6 showed a modest increase compared to control but 20:4, n-6 fell slightly. The n-3 fatty acid species 20:5, n-3 and 22:6, n-3 were similar to control following the n-6 PUFA diet which raised the n-6:n-3 ratio approximately 50% but did not alter the double bond index.

TABLE 7.9 *Fatty acid composition of total phospholipid, including the n-6:n-3 ratio and double bond index (DBI), in hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Fatty acid species,	Diet		
n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
18:2, n-6	16.0±0.3^{a}	16.3±0.8^{b}	19.1±0.7^{a,b}
20:4, n-6	26.6±0.4^{c,d}	22.3±0.7^{c,e}	24.6±0.5^{d,e}
20:5, n-3	-	0.3±0.1	-
22:6, n-3	8.4±0.2^{f}	13.7±1.2^{f,g}	7.5±1.1^{g}
n-6:n-3 ratio	4.6±0.2^{h,i}	2.9±0.6^{h,j}	6.2±0.6^{i,j}
DBI	223±2	233±4	223±2

Values: mean ± s.e.m., n=8 per dietary group. Data for fatty acids represent the percentage weight of total fatty acid species. Superscript characters denote significant differences ($0.001 < p < 0.05$) between individual fatty acid species and parameters in relation to diet.

In fractionated phospholipid (Table 7.10), dietary supplementation with n-3 PUFA did not affect 18:2, n-6 compared to control in any fraction. The fall in 20:4, n-6 seen in total phospholipid in relation to n-3 PUFA occurred to a large extent in PE and PC with a small decrease in PS; 20:4, n-6 in PI was not altered.

TABLE 7.10 *Fatty acid composition of fractionated phospholipid in hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Phospholipid fraction and fatty acid species		Diet		
		Control	n-3 PUFA	n-6 PUFA
PC	18:2, n-6[b,c]	5.7±0.3	5.7±0.2	8.4±0.5
	20:4, n-6[a,c]	34.7±0.4	30.3±0.3	33.8±0.4
	20:5, n-3	-	0.2±0.1	-
	22:6, n-3[a,b,c]	3.5±0.2	7.9±0.3	2.7±0.1
PE	18:2, n-6[b,c]	4.0±0.1	3.8±0.1	5.9±0.2
	20:4, n-6[a,b,c]	27.9±0.4	20.2±0.4	25.5±0.5
	20:5, n-3	-	0.3±0.0	-
	22:6, n-3[a,b,c]	19.5±0.4	31.3±0.4	15.3±0.4
PI	18:2, n-6[b,c]	3.7±0.1	3.6±0.2	6.7±0.2
	20:4, n-6	37.8±0.5	36.1±0.8	36.8±0.6
	20:5, n-3	-	-	-
	22:6, n-3[a,b,c]	1.8±0.1	3.5±0.2	1.3±0.1
PS	18:2, n-6[b,c]	1.6±0.1	1.6±0.1	2.4±0.2
	20:4, n-6[a,b,c]	8.2±0.1[i]	6.6±0.2	7.7±0.1
	20:5, n-3	-	-	-
	22:6, n-3[a,b,c]	15.9±0.7	22.1±0.8	12.2±0.8

Values are mean ± s.e.m. and represent the percentage weight of total fatty acid species, n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between individual fatty acid species within phospholipid fractions in relation to diet. [a] Control *vs* n-3 PUFA, [b] control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA

With dietary n-3 PUFA, 20:5, n-3 was found in PC and PE, where it was not detected (< 0.1%) in relation to the other diets, but not in PI and PS where this fatty acid species was undetectable regardless of diet. Increases in 22:6, n-3 following n-3 PUFA ranged from 25% in PS to greater than 100% in PC and PI. In relation to the n-6 PUFA diet, 18:2, n-6 increased in all fractions but 20:4, n-6 was either unaltered (PC and PI) or actually decreased (PE and PS). Of the n-3 fatty acid species, 20:5, n-3 was not detectable following dietary n-6 PUFA and 22:6, n-3 fell by approximately 25% compared to control.

The n-3 PUFA supplement halved the n-6:n-3 ratio in all fractions and significantly increased the double bond index in PC and PE, but not in PI and PS (Table 7.11). In response to n-6 PUFA, the n-6:n-3 ratios rose by 50% but the double bond index did not change, except in PE where it fell significantly.

TABLE 7.11 *Fractionated phospholipid n-6:n-3 ratios and double bond indices (DBI) in hearts isolated from flurbiprofen-treated rats fed experimental diets.*

Phospholipid fraction,		Diet		
	n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
PC	n-6:n-3 ratio[a,b,c]	9.0±0.4	3.6±0.1	12.8±0.7
	DBI[a,c]	195±1	205±1	195±1
PE	n-6:n-3 ratio[a,b,c]	1.8±0.0	0.8±0.0	2.6±0.1
	DBI[a,b,c]	289±2	307±1	280±3
PI	n-6:n-3 ratio[a,b,c]	15.3±0.7	7.8±0.4	23.6±1.9
	DBI	190±3	194±4	190±2
PS	n-6:n-3 ratio[a,b,c]	1.2±0.1	0.5±0.0	2.0±0.2
	DBI	200±6	204±7	198±6

Values: mean ± s.e.m., n=11 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between parameters for individual phospholipid fractions. [a] Control *vs* n-3 PUFA, [b] control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA

7.3.8 Effect of flurbiprofen on phospholipid fractions and fatty acid composition

Comparison of the above phospholipid results with those presented in Chapter 6, where identical diets were administered to animals which were not treated with flurbiprofen, confirmed that the amount of total phospholipid and the proportions of the individual phospholipid fractions did not change in relation to flurbiprofen. In the phospholipid fractions, analysis of variance revealed that levels of 18:2, n-6 in PC and PI were significantly lower for all three diets following flurbiprofen. Regarding 20:4, n-6, this fatty acid species rose significantly in PC and PE and to a lesser extent in PS, but not in PI. Changes in 22:6, n-3 in relation to flurbiprofen were limited but the increases for all three diets in PI were significant. Although the n-6:n-3 ratio in PC, PE and PS did not alter significantly between the two studies, the double bond indices increased in these three fractions, regardless of diet, following flurbiprofen. In PI, the n-6:n-3 ratio was significantly decreased in PI following flurbiprofen, strikingly so for the control and n-6 PUFA dietary groups where it fell by a third, and the double bond index tended to increase, particularly in relation to the n-6 PUFA diet.

7.3.9 Correlation between LVP responses and fatty acid composition

Regression of peak LVP responses against the fractionated phospholipid fatty acid, n-6:n-3 ratio and double bond index data, singly and in multiple combinations, failed to reveal any significant correlations.

7.4 Discussion

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We found that an n-6 PUFA diet (40% fat calories, P/S ratio 2.0) had no effect on peak LVP responses to α_1-adrenoceptor stimulation in hearts isolated from rats treated with parenteral flurbiprofen for the preceding 48 hours. Dietary supplementation with n-3 PUFA was associated with a non-significant trend to attenuation of peak LVP. Neither the n-6 nor the n-3 PUFA diet affected $LVdP.dt^{-1}_{max}$, $LVdP.dt^{-1}_{min}$, or heart rate compared to control, though heart rates were higher in the n-6 PUFA group compared to the n-3 PUFA group. The

flurbiprofen regimen did not abolish cardiac prostanoid release in the three dietary groups, as reflected by coronary venous levels of 6-*oxo*-$PGF_{1\alpha}$ but did lead to a 75% reduction in release. Both PUFA diets brought about significant changes in cardiac fractionated phospholipid fatty acid composition.

The findings for n-6 PUFA are clearly in contrast to those of Chapter 6. In the absence of flurbiprofen, an identical n-6 PUFA diet was associated with significant attenuation of the peak LVP response to α_1-adrenoceptor stimulation. Interestingly, the overall effects of a small dose of n-3 PUFA were similar in the two studies; a non-significant trend to attenuation of peak LVP responses which does not appear to be susceptible to the added influence of flurbiprofen. There was no tendency in the presence of flurbiprofen for baseline peak LVP to be lower with n-3 PUFA.

As was discussed in Chapter 6, the mechanism underlying the effects of dietary n-6 PUFA on responses in the isolated heart remains unclear. In the present study, the effect of n-6 PUFA on peak LVP was virtually removed by pre-treating the animals with a cyclooxygenase inhibitor. On the basis of similar evidence, it has previously been concluded that enhanced prostanoid release must have some role in n-6 PUFA dietary effects [Hoffman *et al.*, 1982, McLennan *et al.*, 1987]. However, whether with or without flurbiprofen, we found baseline prostacyclin release to be unaltered by diet (see Table 7.6). The possibility remains that dietary PUFA might influence prostanoid release in relation to α_1-adrenoceptor stimulation. We measured coronary venous 6-*oxo*-$PGF_{1\alpha}$ prior to commencing noradrenaline and not during the dose response relationship. Accordingly, we have not excluded the enhancement by dietary n-6 PUFA of prostanoid release in association with α_1-adrenoceptor stimulation, a mechanism consistent with our data. We are not aware of evidence to either support or contest this theory.

The administration of flurbiprofen was also associated with differences in phospholipid fatty acid composition. Levels of 18:2, n-6 were lower, and levels of 20:4, n-6 higher, in relation to flurbiprofen treatment but the degree of change was the same regardless of diet. In contrast to other fractions, the n-6:n-3 ratios in PI appeared

susceptible to the use of flurbiprofen but the ratio for the control and n-6 PUFA diets fell to the same extent, a third, between studies. Thus, the effects of dietary n-6 PUFA are not readily explained by changes in phospholipid fatty acid composition. Once more, no correlations were found between the change in cardiac responses and the change in phospholipid fatty acid composition with respect to diet.

Flurbiprofen did not alter the overall effect of dietary n-3 PUFA in these studies, although any tendency to lower baseline peak LVP disappeared. In both the present and the previous chapter, a non-significant trend to attenuation of LVP was found. This is of the same nature as the significant effect of a considerably higher dose of n-3 PUFA (Chapter 5). Others have found the effects of a similarly extreme n-3 PUFA diet to be resistant to combined cyclooxygenase/lipoxygenase inhibition (Reibel *et al.*, 1988). It would appear that the effects of dietary n-3 PUFA on the responses to α_1-adrenoceptor stimulation in the heart are not mediated by some alteration in prostanoid release.

In summary, the previously documented effects of dietary n-6 PUFA on the responses of the isolated rat heart to α_1-adrenoceptor stimulation were found to be almost entirely removed by pre-treatment of the animals with parenteral flurbiprofen. However, baseline cardiac prostacyclin release, either with or without flurbiprofen treatment, was unaffected by diet. It remains possible that stimulated, rather than baseline prostacyclin release was influenced by dietary n-6 PUFA. The overall effects of n-3 PUFA, albeit not significant, were the same regardless of flurbiprofen treatment.

In the following chapter, the discrepancy between the effects of dietary n-6 PUFA in 40% fat diets in the heart and vasculature is pursued further by examining the effect of these diets on the fractionated phospholipid fatty acid composition of hearts and aortas.

Chapter 8

Dietary polyunsaturated fatty acids and phospholipid fatty acid composition in heart and aorta

8.1 Introduction

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In Chapters 4,6, and 7, we used identical dietary regimens supplying 40% of total calories from fat. The control diet was relatively rich in saturated fat with a P/S ratio of 0.3, representing the fat intake of middle-aged Scottish men. The n-3 PUFA diet was the control diet supplemented with 0.4% dietary calories fish oil. The n-6 PUFA diet had a P/S ratio of 2.0, achieved by increasing the proportion of 18:2, n-6 at the expense of saturated fatty acids.

The n-3 PUFA diet was associated with significant attenuation of the responses to α_1-adrenoceptor stimulation in the isolated resistance artery (Chapter 4) but effects of a similar nature in the heart were not significant (Chapters 6 and 7). Conversely, for the n-6 PUFA diet the effect in the heart was significant (Chapter 6) but not that in the resistance artery.

Analysis of the fatty acid composition of the resistance arteries was not possible but the different effects of diet on vascular and cardiac responses persuaded us to attempt a comparison between the three 40% fat diets in terms of their influence on fractionated phospholipid fatty acid composition in the heart and aorta.

8.2 Methods

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Twenty-four Sprague-Dawley rats were randomly separated into three groups of 8 before commencing the 40% fat calorie semisynthetic diets. Two groups were fed the control P/S ratio 0.3 diet and one of these, the n-3 PUFA group, received fish oil daily by gavage. The third group received the n-6 PUFA P/S ratio 2.0 diet. The control and n-6 PUFA groups received daily control oral doses of olive oil, which had been retained from their dietary fat intake. At the end of the feeding period, hearts were excised and placed in ice-cold buffer (section 2.6.2) until beating stopped. The hearts were then trimmed of atria and great vessels, opened, blotted, weighed and placed in Nunc tubes under liquid nitrogen. Aortas were dissected free from arch to iliac bifurcation and flushed through with ice-cold 0.9% saline before being gently cleaned free of the adjacent inferior vena cava and adherent adipose tissue under 0.9% saline in a petri dish. Aortas were then blotted and frozen in an identical manner to the hearts. Subsequently, the fatty acid composition of fractionated phospholipid was determined (section 2.7.4).

8.3 Results

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8.3.1 Body and heart weights

Neither final body weights nor heart weights varied with respect to diet (Table 8.1).

TABLE 8.1 *Final body weights (g)and heart weights (g) of rats fed experimental diets.*

Diet	Body weight	Heart weight
Control	379±17	0.9±0.0
n-3 PUFA	383±9	1.1±0.1
n-6 PUFA	368±9	1.1±0.1

Values: mean ± s.e.m., n=8 per dietary group. There were no significant differences in relation to diet.

8.3.2 Fatty acid composition of cardiac tissue fractionated phospholipid

As before, the amount of total phospholipid and the distribution of the individual phospholipid fractions in cardiac tissue did not differ with respect to diet (Table 8.2).

TABLE 8.2 *Phospholipid fractions and total phospholipid in the hearts of rats fed experimental diets.*

Phospholipid fraction	Diet		
	Control	n-3 PUFA	n-6 PUFA
PC	4164±669	4062±423	4098±779
PE	2533±652	2627±629	2275±596
PI	441±58	436±38	445±61
PS	261±64	217±47	218±45
Total	10788±1093	10723±1008	11056±1184

Values (mean ± s.e.m.) are expressed as μg.g fatty acids.g wet weight^{-1}, n=8 per dietary group. There were no significant differences in relation to diet.

The fatty acid composition of cardiac fractionated phospholipid demonstrated many statistically significant differences with respect to diet (Table 8.3). The n-3 PUFA diet was associated with small increases in 18:2, n-6 in all fractions but PE. The proportion of 20:4, n-6 was decreased in all fractions following n-3 PUFA but only to a small extent in PI. Of the n-3 fatty acid species, 20:5, n-3 was detected in PC, PI and PS, but not in PI, and only in relation to the n-3 PUFA diet. Levels of 22:6, n-3 rose 50% in PE and PI and were doubled in PC with n-3 PUFA supplementation but were little altered in PS. Following the n-6 PUFA diet, 18:2, n-6 rose in all fractions, being doubled in PI, but 20:4, n-6 fell in all fractions. The n-3 fatty acid species 20:5, n-3 was not detected (< 0.1%) in relation to the n-6 PUFA diet and levels of 22:6, n-3 decreased by approximately 25%.

TABLE 8.3 *Fatty acid composition of phospholipid fractions in the hearts of rats fed experimental diets.*

Phospholipid fraction and fatty acid species		Diet		
		Control	n-3 PUFA	n-6 PUFA
PC	18:2, n-6[a,b,c]	5.3±0.4	7.5±0.4	9.2±0.4
	20:4, n-6[a,b,c]	35.2±0.4	27.9±0.5	33.2±0.5
	20:5, n-3	-	0.3±0.0	-
	22:6, n-3[a,b,c]	3.8±0.2	7.8±0.3	2.5±0.1
PE	18:2, n-6[b,c,]	3.5±0.2	3.7±0.1	6.0±0.2
	20:4, n-6[a,b,c]	27.9±0.6	20.3±0.5	25.6±0.6
	20:5, n-3	-	0.3±0.0	-
	22:6, n-3[a,b,c]	20.9±0.6	30.7±0.5	14.6±0.5
PI	18:2, n-6[a,b,c]	3.5±0.1	4.2±0.1	7.0±0.1
	20:4, n-6[a,b]	38.1±0.7	34.4±0.5	35.8±0.5
	20:5, n-3	-	-	-
	22:6, n-3[a,b,c]	1.8±0.0	3.5±0.1	1.2±0.0
PS	18:2, n-6[a,b,c]	2.0±0.1	2.7±0.2	3.6±0.2
	20:4, n-6[a,c]	9.0±0.3	6.8±0.4	8.5±0.2
	20:5, n-3	-	0.2±0.1	-
	22:6, n-3[b,c]	16.1±0.8	18.8±1.1	11.1±0.5

Values (mean ± s.e.m.) represent the percentage weight of total fatty acids within each phospholipid fraction, n=8 per dietary group. Superscript characters denote significant differences ($0.001 < p < 0.05$) between individual fatty acid species within different phospholipid fractions in relation to diet. [a] Control *vs* n-3 PUFA, [b] Control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA

In response to the n-3 PUFA diet, the n-6:n-3 ratios were halved in PC, PE and PI but fell less in PS, and only in PE was the double bond index significantly increased (Table 8.4). Following n-6 PUFA, the n-6:n-3 ratios rose by 70-75% and the double bond index fell in PC and PE; it did not change in PI and PS.

TABLE 8.4 *Phospholipid fraction n-6:n-3 ratios and double bond indices (DBI) in the hearts of rats fed experimental diets.*

Phospholipid fraction,		Diet		
	n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
PC	n-6:n-3 ratio[a,b,c]	8.1±0.4	3.6±0.2	13.9±0.6
	DBI[b,c]	199±1	199±2	192±1
PE	n-6:n-3 ratio[a,b,c]	1.6±0.1	0.8±0.0	2.7±0.1
	DBI[a,b,c]	289±2	301±1	271±1
PI	n-6:n-3 ratio[a,b,c]	14.2±0.4	7.5±0.2	25.3±1.0
	DBI	193±2	190±2	186±2
PS	n-6:n-3 ratio[b,c]	1.3±0.1	0.9±0.2	2.3±0.1
	DBI	205±6	199±6	193±3

Values: mean ± s.e.m., n=8 per dietary group. Superscript characters denote significant differences ($0.0001 < p < 0.05$) between parameters for individual phospholipid fractions in relation to diet. [a] Control *vs* n-3 PUFA, [b] Control *vs* n-6 PUFA, [c] n-3 PUFA *vs* n-6 PUFA

8.3.3 Fatty acid composition of aortic tissue fractionated phospholipid

It was necessary to pool the tissue from 4 of the 8 aortas from each dietary group in order to have sufficient for fractionated phospholipid analysis. Mean values (effective n=2) for the phospholipid fractions, and the proportion each fraction represented (of PC+PE+PI+PS), are given in Table 8.5. Diet did not affect the relative amounts of the individual phospholipid fractions.

TABLE 8.5 *Phospholipid fractions in the aortas of rats fed experimental diets.*

Phospholipid fraction	Diets		
	Control	n-3 PUFA	n-6 PUFA
PC	738 (49%)	831 (48%)	764 (52%)
PE	509 (34%)	472 (32%)	519 (31%)
PI	127 (8%)	124 (9%)	145 (8%)
PS	139 (9%)	143 (11%)	167 (9%)

Values are means and are expressed as μg fatty acids.g wet weight^{-1}, n=2 results per dietary group. In parentheses is the proportion each fraction represented as a percentage of Σ(PC+PE+PI+PS).

We did not attempt to analyse total phospholipid fatty acid composition in the aortas because of the small amounts of tissue available, but focused on fractionated phospholipid. Means and errors derived from the results for pooled tissue are shown in Table 8.6. With an effective sample size of 2, we felt that statistical comparison of the dietary groups was not appropriate. The following is a qualitative interpretation. In relation to the n-3 PUFA supplement, 18:2, n-6 increased slightly and 20:4, n-6 fell in PC and PE. Of the n-3 fatty acid species, small proportions of 20:5, n-3 were found in all fractions and there were 25% increases in 22:6, n-3. With the n-6 PUFA diet, 18:2, n-6 increased in all fractions but 20:4, n-6 altered little, even falling slightly in PE. Following n-6 PUFA, 20:5, n-3 was not detected (< 0.1%) and 22:6, n-3 fell in PC, PE and PS, though not in PI.

TABLE 8.6 *Fatty acid composition of phospholipid fractions in aortic tissue from rats fed experimental diets.*

Phospholipid fraction and fatty acid species		Diet		
		Control	n-3 PUFA	n-6 PUFA
PC	18:2, n-6	8.1±0.2	10.2±1.2	13.9±0.5
	20:4, n-6	21.8±0.4	16.1±0.7	22.0±0.2
	20:5, n-3	-	0.5±0.1	-
	22:6, n-3	1.3±0.1	2.5±0.6	0.9±0.1
PE	18:2, n-6	3.1±0.2	4.1±1.0	6.7±0.4
	20:4, n-6	38.8±0.2	33.4±2.3	36.5±0.2
	20:5, n-3	-	1.1±0.2	-
	22:6, n-3	5.5±0.1	10.2±1.8	4.0±0.2
PI	18:2, n-6	2.2±0.4	3.0±0.7	4.4±0.4
	20:4, n-6	38.2±2.3	35.0±1.7	37.6±0.6
	20:5, n-3	-	0.3±0.0	-
	22:6, n-3	0.4±0.0	0.9±0.2	0.3±0.0
PS	18:2, n-6	2.5±0.2	3.4±0.1	4.2±0.5
	20:4, n-6	12.2±0.2	10.7±0.5	13.0±0.1
	20:5, n-3	-	0.1±0.1	-
	22:6, n-3	3.3±0.5	5.5±1.6	2.2±0.2

Values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=2 results per dietary group.

The n-6:n-3 ratios and DBI values for the aortic phospholipid fractions are given in Table 8.7. Again, statistical comparison was not performed because of the sample size. Profound changes in n-6:n-3 ratio occurred with both PUFA diets, particularly in the PI fraction. The DBI was less affected by diet but tended to be unaltered or decreased

with n-3 PUFA. There was no consistent effect of n-6 PUFA which increased the DBI in PC and decreased it in PE.

TABLE 8.7 *Phospholipid fraction n-6:n-3 ratios and double bond indices (DBI) in aortic tissue from rats fed experimental diets.*

Phospholipid fraction,		Diet		
	n-6:n-3 ratio and DBI	Control	n-3 PUFA	n-6 PUFA
PC	n-6:n-3 ratio	20.1±0.4	8.3±1.9	34.7±1.1
	DBI	145±1	136±4	151±2
PE	n-6:n-3 ratio	7.8±0.1	3.3±0.6	11.6±0.3
	DBI	264±1	267±3	255±2
PI	n-6:n-3 ratio	71.8±9.5	24.0±4.5	111.5±16.0
	DBI	182±7	176±3	183±1
PS	n-6:n-3 ratio	6.2±0.6	3.1±0.7	10.6±0.7
	DBI	146±8	146±12	146±0.5

Values: mean ± s.e.m., n=2 results per dietary group.

8.3.4 Aortic *versus* cardiac phospholipid fatty acid composition

The small sample sizes for the aortic fractionated phospholipid fatty acid composition results precluded statistical comparison with the cardiac results. However, to assist interpretation, Table 8.8 lists the aortic data from Table 8.6 together with the 95% confidence intervals for the respective cardiac data. Generally, levels of 18:2, n-6 were of a similar order in the two tissues. The proportion of 20:4, n-6, although similar in PI and PS, was substantially lower in aortic PC and increased in PE. The small proportion of 20:5, n-3 was more evident in aortic tissue and was detected in PI. Regarding 22:6, n-3, this fatty acid species was lower in all fractions, substantially so in PE, PI and PS.

TABLE 8.8 *Fatty acid composition of phospholipid fractions in aortic tissue, and (95% confidence intervals) for cardiac tissue results, from rats fed experimental diets.*

Phospholipid fraction and fatty acid species		Diet: Control		n-3 PUFA		n-6 PUFA	
		Aortic	Cardiac	Aortic	Cardiac	Aortic	Cardiac
PC	18:2, n-6	8.1±0.2	(4.4-6.2)	10.2±1.2	(6.7-8.4)	13.9±0.5	(8.2-10.3)
	20:4, n-6	21.8±0.4	(34.3-36.1)	16.1±0.7	(26.7-29.0)	22.0±0.2	(32.0-34.4)
	20:5, n-3	-	(-)	0.5±0.1	(0.2-0.4)	-	(-)
	22:6, n-3	1.3±0.1	(3.4-4.3)	2.5±0.6	(7.1-8.5)	0.9±0.1	(2.2-2.8)
PE	18:2, n-6	3.1±0.2	(3.1-4.0)	4.1±1.0	(3.4-4.0)	6.7±0.4	(5.6-6.5)
	20:4, n-6	38.8±0.2	(26.4-29.4)	33.4±2.3	(19.1-21.5)	36.5±0.2	(24.3-27.0)
	20:5, n-3	-	(-)	1.1±0.2	(0.2-0.4)	-	(-)
	22:6, n-3	5.5±0.1	(19.5-22.3)	10.2±1.8	(29.5-31.8)	4.0±0.2	(13.4-15.9)
PI	18:2, n-6	2.2±0.4	(3.3-3.8)	3.0±0.7	(3.9-4.4)	4.4±0.4	(6.7-7.3)
	20:4, n-6	38.2±2.3	(36.4-39.7)	35.0±1.7	(33.3-35.4)	37.6±0.6	(34.6-37.1)
	20:5, n-3	-	(-)	0.3±0.0	(-)	-	(-)
	22:6, n-3	0.4±0.0	(1.7-1.9)	0.9±0.2	(3.2-3.8)	0.3±0.0	(1.1-1.3)
PS	18:2, n-6	2.5±0.2	(1.9-2.2)	3.4±0.1	(2.3-3.1)	4.2±0.5	(3.2-4.0)
	20:4, n-6	12.2±0.2	(8.3-9.7)	10.7±0.5	(5.8-7.8)	13.0±0.1	(8.0-9.0)
	20:5, n-3	-	(-)	0.1±0.1	(0.0-0.5)	-	(-)
	22:6, n-3	3.3±0.5	(14.2-18.0)	5.5±1.6	(16.3-21.3)	2.2±0.2	(10.0-12.2)

Aortic values (mean ± s.e.m.) represent the percentage weight of total fatty acid species, n=2 per dietary group. In parentheses are the 95% confidence intervals (n=8) for the cardiac fractionated phospholipid content of the same fatty acid species. Shading identifies aortic results (mean) falling within the cardiac 95% CI range.

In the same manner, Table 8.9 displays the n-6:n-3 ratios and DBI values for aortic tissue together with the 95% confidence interval for the cardiac fractionated phospholipid results. The aortic phospholipid n-6:n-3 ratios were all higher, strikingly

so in PI. Interestingly, the DBI values for aortic tissue phospholipids agreed quite closely with the cardiac values in the PI fraction where they were the same for the n-6 PUFA diet.

TABLE 8.9 *Phospholipid fraction n-6:n-3 ratios and double bond indices (DBI) in aortic tissue, and (95% confidence interval) for cardiac tissue results, from rats fed experimental diets.*

Phospholipid fraction, n-6:n-3 ratio and DBI		Diet: Control		n-3 PUFA		n-6 PUFA	
		Aortic	Cardiac	Aortic	Cardiac	Aortic	Cardiac
PC	n-6:n-3 ratio	20.1±0.4	(7.2-9.0)	8.3±1.9	(3.2-3.9)	34.7±1.1	(12.4-15.3)
	DBI	145±1	(197-200)	136±4	(195-203)	151±2	(189-195)
PE	n-6:n-3 ratio	7.8±0.1	(1.4-1.8)	3.3±0.6	(0.7-0.8)	11.6±0.3	(2.4-3.0)
	DBI	264±1	(285-293)	267±3	(298-304)	255±2	(268-274)
PI	n-6:n-3 ratio	71.8±9.5	(13.1-15.2)	24.0±4.5	(6.9-8.0)	111.5±16.0	(23.1-27.6)
	DBI	182±7	(187-198)	176±3	(187-194)	183±1	(181-190)
PS	n-6:n-3 ratio	6.2±0.6	(1.0-1.5)	3.1±0.7	(0.5-1.3)	10.6±0.7	(2.0-2.6)
	DBI	146±8	(191-219)	146±12	(184-214)	146±0.5	(187-200)

Aortic values: mean ± s.e.m., n=2 results per dietary group. In parentheses are the 95% confidence intervals (n=8) for the cardiac fractionated phospholipid analyses. Shading identifies aortic results (mean) falling within the cardiac 95% CI range.

8.4 Discussion

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Widespread changes in the fractionated phospholipid fatty acid composition of aortic and cardiac tissue were seen in relation to dietary PUFA. Although limited statistically by the small effective sample size for the aortic results, there were clearly quite remarkable differences in the fatty acid composition of the two tissues. Levels of 18:2, n-6 were comparable but 20:4, n-6 was increased in aortic PE and strikingly decreased in aortic PC. There tended to be more 20:5, n-3 in the aortic phospholipid fractions

including in PI, where 20:5, n-3 is usually not detected in the heart. Conversely, levels of 22:6, n-3 were lower in all the aortic fractions, irrespective of diet. These differences were reflected in the n-6:n-3 ratios which were higher in aortic tissue. Also, generally lower DBI values in aortic tissue were further evidence of a lesser degree of incorporation of the longer chain unsaturated fatty acid species.

Published data regarding the influence of diet on the fatty acid composition of vascular and cardiac tissue are sparse. In marmosets, long term feeding with approximately 17% fat calorie diets rich in n-6 PUFA, n-3 PUFA or saturated fat was shown to have a number of small effects on aortic *versus* cardiac total phospholipid fatty acid composition [Charnock *et al.*, 1992]. Aortic 18:2, n-6 levels were 30% lower than cardiac levels in relation to n-6 PUFA and saturated fat, and aortic 22:6, n-3 was 60% lower than cardiac following n-3 PUFA. Otherwise, the fatty acid composition of the heart and aorta were remarkably similar. The diets used by Charnock *et al.* are obviously different from our 40% fat calorie diets but there are clearly considerable differences between the rat and the marmoset with respect to the fatty acid composition of aortic and cardiac tissue and the influence of diet.

In summary, although the present study was limited by the small effective sample size of the aortic data, we found potentially important differences in the fractionated phospholipid fatty acid composition of the heart and aorta in relation to our 40% fat calorie semisynthetic diets.

Chapter 9

Discussion

9.1 Principal findings

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The aim of these studies was to examine the effect of dietary PUFA on vascular and cardiac responses to α_1-adrenoceptor stimulation. With the exception of one series of experiments (Chapter 5), semisynthetic diets deriving 40% of total dietary calories from fat were used in order to represent what is, or could be, consumed by man.

In the perfused hind-quarters (Chapter 3) and isolated resistance artery (Chapter 4), a small dietary supplement of n-3 PUFA significantly attenuated responses to α_1-adrenoceptor stimulation. Dietary n-6 PUFA did not exert this effect, although there was a trend to attenuation in the resistance artery. Neither n-3 PUFA nor n-6 PUFA had any effect on the relaxation to acetylcholine of resistance arteries constricted to 60% of their maximum response. In these vascular studies, the fatty acid composition of the target tissue could not be determined, therefore the influence of the diets in this respect was monitored in cardiac tissue. The fatty acid composition of cardiac fractionated phospholipid revealed widespread changes, which were more extensive following dietary n-3 PUFA, in relation to diet.

Diets deriving 10% of total calories from fat, thus of limited relevance to man, were used in an attempt to corroborate published results [Reibel *et al.*, 1988] and to establish the parameter(s) susceptible to dietary influence in the isolated rat heart during α_1-adrenoceptor stimulation (Chapter 5). Baseline and stimulated peak LVP responses were attenuated by a relatively high dose of n-3 PUFA (3.5% of total dietary calories). An n-6 PUFA enriched diet did not exert this effect, indeed, a small

trend to enhancement of peak LVP was observed. Other cardiac parameters in this model; $LVdP.dt^{-1}$, heart rate and coronary flow, were unaffected by diet. Following a 6-hydroxy-dopamine regimen which has been shown to ablate sympathetic nerve endings [Allely, 1983], and which reduced cardiac noradrenaline content to $\leq 0.6\%$ of control levels in our experiments, the effect of the n-3 PUFA diet on baseline peak LVP disappeared. The attenuation of responses to α_1-adrenoceptor stimulation was maintained.

When we investigated the effects of the 40% fat calorie diets relevant to man on responses in the isolated heart (Chapter 6), the results differed to those found with the 10% fat calorie diets (Chapter 5). Dietary n-6 PUFA now brought about the significant attenuation of peak LVP responses to α_1-adrenoceptor stimulation whereas a small n-3 PUFA dietary supplement did not. The effect of n-3 PUFA was curtailed to a non-significant trend to attenuation. Subsequently, the attenuatory effect of n-6 PUFA was shown to be removed by treating the animals with parenteral flurbiprofen (Chapter 7). The trend seen with n-3 PUFA remained. Baseline coronary venous 6-*oxo*-$PGF_{1\alpha}$ concentrations, reflecting prostacyclin release, were reduced 75% by flurbiprofen but were not influenced by diet. In neither of these consecutive studies could fractionated phospholipid fatty acid composition, which was altered by diet and further affected in relation to flurbiprofen treatment, be correlated with peak LVP.

Generally, the changes in phospholipid fatty acid composition following the administration of n-3 PUFA were most evident in PC and PE where 18:2, n-6 and 20:4, n-6 were reduced and 20:5, n-3 and 22:6, n-3 increased. In association with these changes, the n-6:n-3 ratios fell and the double bond indices rose. In contrast, the effects of dietary n-6 PUFA were essentially limited to modest increases in 18:2, n-6 and in the n-6:n-3 ratio; levels of 20:4, n-6 and the double bond index were either unaltered or fell. In relation to the 40% fat calorie diets, PI, and to some extent PS, proved relatively resistant to the influence of diet. This was particularly true of 20:4, n-6 in PI which was tightly conserved.

In view of the differences between the effects of the 40% fat calorie diets in the vascular preparations and the isolated heart, a further study was performed to examine the changes in phospholipid fatty acid composition in the aorta and heart following these diets. Although this study was limited by the need to pool aortic tissue in order to have sufficient for analysis, striking differences were apparent in fatty acid composition between the two tissues. Aortic PC incorporated less 20:4, n-6, and aortic PE more, than the heart. Noticeably, all aortic fractions contained more 20:5, n-3, but less 22:6, n-3, than the heart. Levels of 18:2, n-6 were broadly similar. Further evidence of lesser incorporation of long chain unsaturated fatty acid species in the aorta compared to the heart were higher n-6:n-3 ratios and lower double bond indices.

9.2 Limitations to the studies

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The difficulty in measuring the fatty acid composition of vascular fractionated phospholipid was confirmed in Chapter 8. Despite removing the entire aorta, the tissue from 4 animals was required for a single analysis. Nevertheless, it is clear that the fatty acid composition of vascular tissue is inadequately reflected by that of cardiac tissue. The usefulness of the fatty acid data presented in Chapters 3 and 4 is limited to verifying that the diets administered were capable of inducing significant changes in phospholipid fatty acid composition after 8 weeks feeding.

In the isolated heart studies, there were no direct relationships between peak LVP, which was altered by diet, and cardiac phospholipid fatty acid composition. Accordingly, the discussion of potential mechanisms in the effects observed must draw on the experimental data of other investigators, and is therefore speculative.

It has become widely recognised that dietary n-3 PUFA can influence vascular reactivity by enhancing endothelium-dependent relaxation [Shimokawa *et al.*, 1987, Yin *et al.*, 1988, Yin *et al.*, 1991]. The studies of Chapter 4 do not fully address these possibilities, referred to in greater detail below (section 9.3). Likewise, effects of n-3 PUFA on the coronary arterial endothelium, and possibly the endocardium, may have

contributed to the effects on peak LVP shown in Chapter 5. This is discussed in section 9.4.

The physiological relevance of observations made in the retrogradely perfused isolated heart is limited. In the light of the effects on peak LVP, and lack of effects on $LVdP.dt^{-1}$ and heart rate, it would be potentially rewarding to pursue the effects of dietary PUFA further in the working heart so that a more comprehensive study of effects on contractility could be performed.

Perfusing the hind-quarters and the isolated heart with crystalloid buffer obviously removed any potential interaction with blood elements. While this simplifies the interpretation of the phenomena observed, it would be interesting to investigate dietary effects in blood perfused systems in view of, for example, the increased erythrocyte deformability and decreased whole blood viscosity reported in relation to n-3 PUFA [Terano *et al.*, 1983, Cartwright *et al.*, 1985, Rogers *et al.*, 1987].

9.3 Dietary PUFA and vascular responses

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The significant effects of dietary n-3 PUFA on responses to α_1-adrenoceptor stimulation in the perfused hind-quarters and isolated resistance artery (Chapters 3 and 4) may have been indirect. It has been proposed that similar effects of n-3 PUFA in other studies were due to a decrease in the synthesis and release of vasoconstrictor prostanoids, PGH_2 and TXA_2 [Lockette *et al.*, 1982, Yin *et al.*, 1991]. However, these prostanoids constitute a very small fraction (approximately 2%) of the prostanoids released by rat vascular tissue. The vasoconstrictor $PGF_{2\alpha}$ is released following vasoconstriction induced by KCl but not in response to noradrenaline, unlike the vasodilators PGI_2 and PGE_2 [Pipili and Poyser, 1981]. Indeed, despite the changes in aortic phospholipid fatty acid composition it induced (increased 20:5, n-3 and reduced PC 20:4, n-6), the n-3 PUFA supplement may have led to increased release of vasodilator prostanoids in relation to α_1-adrenoceptor stimulation. It is usually assumed that PGI_2 is simply replaced by PGI_3, an equiactive vasodilator,

following dietary n-3 PUFA [Fischer and Weber, 1984]. However, it has been shown in man that n-3 PUFA can increase PGI_3 synthesis without compromising PGI_2 [von Schacky *et al.*, 1985*b*] and, recently, that n-3 PUFA can produce a paradoxical increase in vascular PGI_2 synthesis [de Caterina *et al.*, 1990]. Thus a net gain in vasodilator prostanoid release, rather than a loss, can occur with n-3 PUFA. Dietary n-6 PUFA did not exert significant effects in these studies and the trend to attenuation of contraction in the resistance artery is unexplained. Despite the lack of influence of the n-6 PUFA diet on aortic phospholipid 20:4, n-6 content, the results of Chapters 6 and 7 suggest that a prostacyclin-related mechanism was responsible. Further studies with cyclooxygenase inhibitors are necessary to clarify these issues with regard to both n-3 and n-6 PUFA.

The possibility that dietary n-3 PUFA was associated with enhanced endothelium-dependent relaxation in the vascular studies was raised (Chapters 3 and 4). Tonic release of EDRF may be important [Malta *et al.*, 1986, Martin *et al.*, 1986, Bullock *et al.*, 1986] but appreciable effects on baseline relaxation state in our studies seem unlikely in view of the normalisation results in the isolated resistance artery. EDRF is released by noradrenaline, but probably through endothelial α_2-adrenoceptor activation [Cocks and Angus, 1983, Miller *et al.*, 1984, Bullock *et al.*, 1986] and the α_2-adrenoceptor antagonists rauwolscine and yohimbine were present in our studies. Difficult to exclude, particularly in the resistance artery where the n-3 PUFA effect was evident at higher levels of active tension, is enhanced release of EDRF in response to vascular stress [Hutcheson and Griffith, 1991, Lamontagne *et al.*, 1992]. This could have contributed to our findings. Lastly, it appears that dietary n-3 PUFA in higher doses may enhance vascular relaxation by an endothelium independent mechanism [Malis *et al.*, 1991].

These possibilities could be addressed by including the use of L-arginine analogues or methylene blue, and de-endothelialised vessels, in future resistance artery studies.

9.4 Dietary PUFA and cardiac responses

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A relatively high dose of n-3 PUFA (3.0% total dietary calories) in a 10% fat calorie diet reduced peak LVP at baseline and during α_1-adrenoceptor stimulation in the isolated heart (Chapter 5). The baseline effect was not present in chemically denervated animals, suggesting that spontaneous noradrenaline release is normally attenuated by dietary n-3 PUFA in this particular model. The changes in cardiac fractionated phospholipid fatty acid composition associated with the 10% fat calorie n-3 PUFA diet were profound. There were substantial reductions in 20:4, n-6 which extended to the PI fraction, usually resistant to dietary influence. Although there was no correlation between fatty acid composition and peak LVP, it is possible that this diet exerted direct effects, discussed fully in section 9.5, on α_1-adrenoceptor function. Alternatively, cardiac prostanoid synthesis, considered below, may have been affected by the reductions in 20:4, n-6.

The potential effects of n-3 PUFA on EDRF and the endocardium should be considered. EDRF is released from the coronary arteries [Collins *et al.*, 1986, Shimokawa *et al.*, 1989, Nyborg, 1990, Kelm *et al.*, 1991, Lamontagne *et al.*, 1992] and the endocardium [Shah *et al.*, 1991]. If the actions of EDRF, which can reduce contractility, were enhanced by n-3 PUFA, this might have contributed to the attenuation of peak LVP. However, in isolated papillary muscles, EDRF reduces peak tension by accelerating relaxation and shortening the duration of contraction [Shah *et al.*, 1991, Henderson *et al.*, 1992]. Translated to the isolated heart, an increase in $LVdP.dt^{-1}_{min}$ would be anticipated and we did not find any effect of diet on $LVdP.dt^{-1}$. Conversely, if dietary n-3 PUFA attenuated the tonic effect of the endocardium, which is an increase in contractility [Brutsaert *et al.*, 1988, Brutsaert 1989] possibly mediated by the putative transmitter "endocardin" [Smith *et al.*, 1991], $LVdP.dt^{-1}_{min}$ and perhaps $LVdP.dt^{-1}_{max}$ would be expected to decrease. To investigate combined effects of n-3 PUFA on both EDRF and the endocardium,

further experimental studies, using inhibitors of EDRF and endocardial disruption, are required.

Compared to the 10% fat calorie diets, the effects on the isolated heart of the 40% fat calorie diets relevant to man were quite different (Chapters 6 and 7). The n-6 PUFA diet (P/S ratio 2.0) significantly attenuated peak LVP responses to α_1-adrenoceptor stimulation whereas the n-3 PUFA supplement was associated with a non-significant trend to attenuation. The changes in fractionated phospholipid fatty acid composition following these diets were less striking than for the 10% fat diets which may account for the lesser n-3 PUFA effect. The n-6 PUFA effect was unexplained. Again, there were no significant correlations between fatty acid composition and peak LVP. Following parenteral flurbiprofen, a cyclooxygenase inhibitor, which reduced baseline PGI_2 release by 75%, the n-6 PUFA effect on peak LVP was no longer apparent. The n-3 PUFA trend remained, indicating that the effect of n-3 PUFA on cardiac responses is independent of prostanoid synthesis and release. Baseline PGI_2 release (PGI_2 is the principal prostanoid released from the isolated the rat heart [de Deckere *et al.*, 1977]) was not influenced by diet but the results suggest that prostanoid release during α_1-adrenoceptor stimulation, which we did not measure, differed for the n-6 PUFA group. Unfortunately, the inotropic actions of prostanoids are controversial. In the isolated rat heart and atrium, PGI_2 has been shown to be a positive inotrope and to shorten the contraction/relaxation cycle [Shaffer and Malik, 1984, Metsae-Ketelae 1981]. In contrast, studies in rat cardiomyocytes have revealed negatively inotropic effects of PGI_2 and PGE_2 [Auclair *et al.*, 1988] and biphasic effects of PGI_2; positively inotropic at low concentrations and negatively inotropic at higher concentrations [Das *et al.*, 1983]. In the guinea-pig heart, positively inotropic actions of PGI_2 were abolished by the dihydropyridine calcium channel antagonist, nifedipine [Fassina *et al.*, 1983], which supports the finding that PGI_2 increased the inward Ca^{2+} current in guinea pig cardiomyocytes [Alloatti *et al.*, 1991]. It is clear that the attenuation of peak LVP by dietary n-6 PUFA was a prostanoid-*related* phenomenon, but as the inotropic actions of PGI_2

remain uncertain, we cannot deduce whether prostanoid synthesis was enhanced or depressed by this diet. Further studies measuring prostanoid release during α_1-adrenoceptor stimulation in our model are necessary to clarify this issue.

9.5 Dietary PUFA, membrane enzyme function and signalling

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By means of steady state fluorescence polarization, both n-3 and n-6 PUFA have been shown to increase membrane fluidity in the sarcoplasmic reticulum [Abeywardena *et al.*, 1984] and platelets [Hornstra and Rand, 1986] of diet-fed rats. Also, a 5% fat diet (P/S ratio 1.0), as opposed to a fat-deficient diet, led to greater membrane unsaturation associated with a lower gel-liquid phase transition temperature [King *et al.*, 1977]. Alterations in the biophysical properties of the cell membrane, as would be expected with the experimental diets used in our studies, may affect the function of membrane-bound enzymes, such as protein kinase C, ATPases and phospholipases. The activation of protein kinase C is thought to be facilitated by membrane unsaturation, which, by widening phospholipid head-group spacing, allows deeper seating of the enzyme in the membrane and encourages conformational change [Epand and Lester, 1990]. Studies in erythrocyte ghosts from rats fed n-6 PUFA diets revealed decreased Na^+/K^+ ATPase activity [Bloj *et al.*, 1973] and increased Ca^{2+}/Mg^{2+} ATPase activity [Holmes *et al.*, 1983], but these effects may not be straightforward or applicable to other tissues and subcellular fractions. The study of Holmes *et al.* [1983] found also that n-6 essential fatty acid deficiency enhanced the activity of Na^+/K^+ and Ca^{2+}/Mg^{2+} ATPases while others report that sarcoplasmic reticulum ATPase activity is unaffected by changes in membrane saturation [Abeywardena *et al.*, 1984]. Effects of PUFA on phospholipase function are interesting because changes in membrane fatty acid composition affect both the environment and the substrate of the enzyme. Dietary n-6 PUFA increased the hydrolysis of PC and PE in rat cardiac membranes [Leonardi *et al.*, 1987] and incubation of rat cardiomyocytes with n-6 PUFA increased the activity of phospholipase A [Nalbone *et al.*, 1990]. Likewise, phospholipase A_2

activity was enhanced in gastric tissue [Grataroli *et al.*, 1988] and renal cortical mitochondria [Malis *et al.*, 1990] from rats fed n-3 PUFA rather than saturated fat.

Recent evidence suggests that cell signalling may be susceptible to n-3 PUFA. The generation of inositol-*tris*-phosphate (IP_3) was found depressed in platelets from rabbits fed n-3 PUFA as opposed to n-6 or n-9 PUFA [Medini *et al.*, 1990]. Incubation of rabbit platelets with 20:5, n-3 reduced the production of IP, IP_2 and IP_3, reflected by the incorporation of ^{3}H-inositol, in response to a thromboxane analogue although there was no detectable increase in platelet phospholipid 20:5, n-3 content [Chetty *et al.*, 1989]. These authors proposed that minute changes in membrane fatty acid composition were sufficient to affect signal transduction and that 20:5, n-3 might attenuate the activity of phospholipase C. Remarkably, these findings are consistent with the results of cell culture studies where extreme fluctuations in fatty acid composition are induced by incubation with PUFA. Vascular smooth muscle cell IP_3 synthesis in response to low density lipoprotein was reduced by culture in medium containing 20:5, n-3 [Locher *et al.*, 1988, Locher *et al.*, 1989] and the addition of 22:6, n-3 to rat cardiomyocyte culture medium caused a decrease in phospholipid ^{3}H-inositol incorporation [Bordoni *et al.*, 1990].

From the above reports, it appears that dietary PUFA, particularly n-6 PUFA, can enhance phospholipase A_2 activity. This would be expected to result in increased prostanoid synthesis where levels of 20:4, n-6 are maintained. On the other hand, n-3 PUFA seem able to compromise the PI signalling pathway regardless of the extent of change in phospholipid n-3 content.

If fluctuations in cell membrane fatty acid composition affect the processes discussed above, then we may speculate that other membrane-linked phenomena, such as α_1-adrenoceptor affinity and receptor/G protein coupling, may be susceptible to the influence of diet. The affinity of β-adrenoceptors for the non-selective antagonist dihydroalprenolol was reduced by n-6 PUFA, as was basal, noradrenaline- and fluoride-stimulated adenylate cyclase activity [Wince and Rutledge, 1981]. These results indicate that receptor and enzyme function, but not G protein function, was

affected by n-6 PUFA in these studies. The potential effects of diet on α_1-adrenoceptor affinity await investigation.

9.6 Conclusions

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In relation to 40% fat calorie diets, which we contend are relevant to man, a small amount of n-3 PUFA significantly attenuated vascular responses, and tended to attenuate cardiac responses, to α_1-adrenoceptor stimulation. The precise rôles of a number of potential mechanisms, suggested by fractionated phospholipid fatty acid data, in this n-3 PUFA effect remain to be clarified. In contrast, dietary n-6 PUFA did not influence vascular responses but attenuated cardiac responses to α_1-adrenoceptor stimulation. This effect of n-6 PUFA was dependent upon prostanoid synthesis. The influence of n-3 PUFA on vascular responses may help to explain the putative BP lowering effects of fish oil in man.

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Appendix 1

Semisynthetic diets: ingredients

§

1.1 40% fat calorie diets

For a 2 kg batch of 40% fat calorie diet, the following were combined.

cornflour	912 g
casein	534 g
cellulose	130 g
fat mixture	370 g
salt mixture	46 g
vitamin mixture	8 g

1.2 10% fat calorie diets

For a 1 kg batch of 10% fat calorie diet, the following were combined.

cornflour	660 g
casein	220 g
cellulose	53 g
fat mixture	92 g
salt mixture	19 g
vitamin mixture	4 g

Appendix 2

Semisynthetic diets: fat mixtures

§

2.1 40% fat calorie diets

The fat mixtures for the P/S 0.3 (40% fat calories) and P/S 2.0 diets were composed of the following (2 kg batch).

P/S 0.3	beef dripping	1485 g
	olive oil	270 g
	safflower oil	245 g
	d-α-tocopherol	574 mg
P/S 2.0	beef dripping	261 g
	olive oil	472 g
	corn oil	1267 g
	d-α-tocopherol	50 mg

2.2 10% fat calorie diets

The fat mixtures for the P/S 0.3 (10% fat calories), n-3 PUFA enriched and n-6 PUFA enriched diets were composed of the following (1 kg batch).

P/S 0.3	beef dripping	743 g
	olive oil	135 g
	safflower oil	122 g
	d-α-tocopherol	1.5 g
n-3 PUFA	fish oil (MaxEPA)	725 g
	safflower oil	275 g
n-6 PUFA	corn oil	1000 g
	d-α-tocopherol	1.4 g

Appendix 3

Semisynthetic diets: salt mixture

§

The dietary salt mixture for all the diets was prepared from the following salts.

calcium carbonate ($CaCO_3$) ..... 1007 g

copper chloride ($CuCl_2.2H_2O$) ..... 2.35 g

ferric citrate ($C_6H_5O_7Fe.3H_2O$) ..... 21.95 g

magnesium hydrogen phosphate ($MgHPO_4.3H_2O$ ..... 478 g

manganese sulphate ($MnSO_4$) ..... 33.9 g

trisodium citrate ($C_6H_5O_7Na_3$) ..... 355.5 g

potassium bicarbonate ($KHCO_3$) ..... 359.5 g

potassium chloride (KCl) ..... 175 g

potassium dihydrogen phosphate (KH_2PO_4) ..... 237.5 g

potassium iodate (KIO_3) ..... 0.035 g

zinc chloride ($ZnCl_2$) ..... 6.25 g

Appendix 4

Semisynthetic diets: vitamin mixture

§

The dietary vitamin mixture for all the diets was prepared from the following compounds.

calcium pantothenic acid ..... 7.5 g

choline chloride..... 375 g

folic acid .....0.375 g

fumed silica..... 75 g

menadione.....0.375 g

myoinositol ..... 37.5 g

nicotinic acid..... 7.5 g

sucrose ..... 991 g

vitamin A (retinol acetate).....0.558 g

vitamin B_1 (thiamine hydrochloride)..... 2.25 g

vitamin B_2 (riboflavin) ..... 2.25 g

vitamin B_6 (pyridoxine hydrochloride) ..... 0.75 g

vitamin B_{12} (cyanocobalamin) .....0.008 g

vitamin D_2 (calciferol) ..... 0.14 g

vitamin H (*d*-biotin) .....0.075 g