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N-3 polyunsaturated fatty acid supplementation alters inositol phosphate metabolism and protein kinase C activity in adult porcine cardiac myocytes

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Abstract

Several mechanisms have been proposed to explain the anti-arrhythmic effects of n-3 polyunsaturated fatty acids. One mechanism is the effect of modifying cell membrane phospholipid and their subsequent effect on intracellular cell signaling via the second messengers, Ins(1,4,5)P_3 and diacylglycerol. Isolated cardiac myocytes from adult pig hearts were used to investigate the effect of n-3 polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, on the inositol phosphate metabolism and protein kinase C activity. Adult porcine cardiac myocytes were grown in media supplemented with 400 μM arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid. After 24 hr, fatty acid analyses of total lipids by TLC in supplemented cells showed that eicosapentaenoic acid and docosahexaenoic acid were selectively incorporated into the phosphatidylinositol fraction. In the diacylglycerol fraction, there was a small incorporation of both eicosapentaenoic acid and docosahexaenoic acid but it was not significantly different from that of controls. To study the effect of membrane phospholipid modification on the phospholipase C mediated inositol lipid cycle, cardiac myocytes were labeled with 4μCi/ml myo-[2-^{3}H]Ins for 48 hr. After stimulation with epinephrine and phenylephrine (α-receptor agonist) the water soluble [^{3}H]Ins products were separated by chromatography on Dowex AG 1-X8 and measured by scintillation counting. After stimulation, the levels of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 in eicosapentaenoic acid and docosahexaenoic acid supplemented myocytes were significantly reduced ($P < 0.05$) compared to arachidonic acid supplemented myocytes. Similarly, eicosapentaenoic acid and docosahexaenoic acid supplemented cells had reduced levels of protein kinase C activity after stimulation compared to arachidonic acid supplemented cells. From these experiments, it is evident that n-3 PUFA supplementation modulates intracellular cell signaling suggesting a possible anti-arrhythmic mechanism.

Keywords: n-3 PUFA; Cell signaling; Cardiac myocytes; PKC

1. Introduction

The role of dietary n-3 PUFA in the prevention of cardiac arrhythmia in animal models and in cardiac myocytes is now well established [1–4]. The mechanisms by which these dietary n-3 PUFA could confer their protective effects have been extensively reviewed [5,6,7]. One of the mechanisms suggested is the modification of membrane phospholipids after n-3 PUFA supplementation and the effect of this modification on intracellular calcium release and calcium channels via the second messenger operated signaling system.

Cardiac membrane phospholipids play a central role in electric signal generation and conduction as receptors involved in cell signaling, transporters and enzymes are embedded in the membrane bilayer, and any change to the fatty acid composition of this membrane could affect their functions. Several phospholipids are involved directly in intracellular signaling pathways. Hydrolysis of membrane phospholipids such as PtdIns [4,5]P_2 (PIP_2) generates second messengers, Ins [1,4,5]P_3 (IP_3) and diacylglycerol (DAG). IP_3 regulates intracellular calcium both by mobilizing calcium from internal stores and possibly by stimulating calcium entry [8]. Ca^{2+} concentration within the myocyte is an important determinant of myocardial contraction and relax-

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ation [9]. While IP_3 mobilizes Ca^{2+} from intracellular stores, DAG, in turn activates PKC that can phosphorylate a spectrum of cardiac proteins that control myocardial excitability and contraction [10]. For the cardiac myocyte, activation of PKC is important for the maintenance of cardiac electrophysiology and contractility [11]. Other phospholipids also have roles in activating or stabilizing enzymes involved in intracellular signaling, for example, phosphatidylserine (PS) is required for PKC activation. All these phospholipids contain fatty acids attached to the *sn*-1 and -2 positions of the glycerol moiety. So it is probable that changing the type of fatty acid present may alter the precise properties of these compounds with regard to their functions in signal transduction [12].

In our previous study [13], we found that when cardiac myocytes were supplemented with AA, EPA or DHA, the n-3 PUFA selectively modified two important lipid fractions, the non-esterified fatty acid (NEFA) and phosphatidylinositol (PI) which have been implicated in the mechanisms of prevention of cardiac arrhythmias.

There have been very few studies using adult cardiac myocytes to investigate the effect of n-3 PUFA supplementation and most of them have been in isolated neonatal ventricular cardiac myocytes from rat. We have used cardiac myocytes isolated from pig hearts because the pig has a similar cardiac physiology and fatty acid metabolism to humans [14]. This study was carried out to determine how the modification of the PI fraction by n-3 PUFA affects subsequent phospholipid mediated responses within the cardiac myocytes. The aim of this study was to therefore study the effect of n-3 PUFA supplementation on the IP_3 and DAG signaling pathways via modification of the phospholipid fatty acid composition.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) unless stated otherwise. For lipid analysis, fatty acid methyl ester standards were purchased from NuCheck Prep (Elysian, MN) and silica gel G plates from Alltech (Deerfield, IL). All solvents were of high performance liquid chromatography grade.

2.1. Cell culture

Freshly harvested adult pig hearts were obtained from a local abbatoir (F.C.Nicholls, NSW, Australia). Cardiac myocytes were isolated from ventricles of adult pigs using the method of Spinale et al. [15] with some modifications [13]. After the ventricles were excised from the heart, they were washed free of blood using a Modified Krebbs (MK) solution (composition in mmol/L: NaCl 145, KCl 5, NaH_2PO_4 1.2, HEPES 20, glucose 10, taurine 30, succinate 5, L-glutamine 25, adenosine 1 and creatine 5). The tissue was then dissociated in two successive enzyme treatments. The first incubation was carried out at 37°C with gentle shaking in a water bath using collagenase (0.11 mg/ml, Sigma, MO, St. Louis). After 35 min the supernatant was discarded and the tissue minced very finely. This was followed by the second incubation in solution containing collagenase (0.11 mg/ml), DNase (type II, 50 Kunitz/ml, Sigma, MO, St. Louis), 300 μM $CaCl_2$ and 2% BSA for 15 min in similar conditions as the first incubation. The tissue and solution were filtered, centrifuged and the pellet resuspended in Dulbecco's Modified Eagle Medium (DMEM, CSL, NSW, Australia) supplemented with 10% (vol/vol) fetal calf serum (FCS) and 2.0 mM Ca^{2+}.

The number of viable cells were counted at 100x magnification by trypan blue exclusion in a hemocytometer. The cell preparation was >90% pure. Only cells that were rod shaped and excluded trypan blue were defined as viable. Proportion of viable cells were between 88 and 90% in each cell culture. The cells were plated at a live cell density of 1.5×10^6 cells/ml and cultured in a tissue culture incubator at 37°C (5% $C0_2$, 95% humidity) for 48–72 hr until the cells had all adhered.

2.2. Fatty acid incubations

After 5–6 days in culture the plates containing cells were divided into four groups. The cells in each group were incubated for 24 hr in medium (DMEM containing 10% FCS) supplemented with either 400 μM arachidonic acid (AA), 400 μM eicosapentaenoic acid (EPA) or 400 μM docosahexaenoic acid (DHA). The fourth group of cells were incubated in unsupplemented DMEM and treated as the control group. The fatty acids used for the incubations were obtained from Sigma (MO, St. Louis) and were >99% pure. They were dissolved in a small volume of ethanol before addition into medium. The final concentration of ethanol in the medium was 0.04%.

Before commencement of this study, a number of experiments were conducted to determine the concentration of fatty acids required for incubation as well as the length of incubation time to ensure maximum incorporation in the adult myocytes. Fatty acid concentrations of 50 μM, 100 μM and 250 μM in the incubation medium were not enough to enrich the myocytes even after 48 hr. Increasing the concentration of the fatty acids to 400 μM ensured a significant incorporation (compared to controls) in only 24 hr.

2.3. Fatty acid analyses

Fatty acid composition of cellular DAG was determined after extraction of lipid from the cells [16] and separation by thin layer chromatography. Briefly, lipid was extracted with chloroform/methanol (2:1,v/v) containing butylated hydroxy toluene (BHT, 0.005% w/v) to prevent oxidation. The samples were dried under nitrogen, reconstituted in a small volume of chloroform and separated on TLC plates (Silica gel 'G', Alltech, NSW, Australia) using a solvent system

comprising hexane/diethyl ether/acetic acid (85:15:1, by vol.) [17]. After spraying the plates with 2,7 dichlorofluoresceine (0.1% w/v) in methanol (Ajax Chemicals, NSW, Australia) for identification, the 1,2 DAG band was scraped from the plates and redissolved in hexane. The fractions were methylated using BF_3-methanol (14% w/v, Sigma, MO, St. Louis) for 40–60 mins at 100°C [18] and analyzed by GLC.

2.4. Gas chromatographic analysis

A 30 m × 0.25 mm (DB-225) fused carbon-silica column, coated with cyanopropylphenyl (J & W Scientific, Folsom, CA) and employing a method modified from Garg and Blake [19] was used to analyze the derivatised fatty acids. The injector and the detector port temperatures were set at 250°C. The oven temperature was initially held at 170°C for 2 min, increased 10°C/min to 190°C, held for 1 min, then increased 3°C/min up to 220°C and maintained for 15 min to give a total run time of 30 min. A split ratio of 10:1 and an injection volume of 5 μl was used. The chromatograph was equipped with a flame ionization detector, autosampler and autodetector. Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of an authentic standard mixture of fatty acid methyl esters and quantified using a Hewlett Packard (NSW, Australia) 6890 Series Gas Chromatograph with Chemstations Version A.04.02 software for GC analysis.

2.5. ^{3}[H] Ins labeling of cardiac myocytes

To label cells, the incubating DMEM was removed and replaced with medium 199 (a low inositol medium) containing 4 μCi/ml myo-[2-^{3}H]Inositol (Amrad Biotech, NSW, Australia) and incubated for 48–60 hr. The incubating media also contained fetal calf serum and antibiotics. After the labeling period, the medium was removed and cells washed twice for 5 min in Hepes-buffered solution. For α-receptor stimulation, the cells were incubated for 30 min at 37°C in Hepes-buffered solution containing 0.5mM epinephrine and 0.5 mM α_1-agonist phenylephrine in the presence of 10 mM lithium chloride. Release of inositol phosphates was terminated by the addition of ice cold 5% trichloro acetic acid (TCA) to the cells.

For stimulation experiments the concentrations of epinephrine, phenylephrine and propranolol were determined after conducting studies in porcine cardiac myocytes to determine their EC_{50} values.

2.6. Separation and measurement of ^{3}[H] labeled inositol phosphates

After terminating inositol phosphate release, cells were centrifuged to remove TCA insoluble material. To remove excess TCA from the extract, the supernatant was re- extracted with 3–4 washes of diethyl ether. The extracts were then neutralized to pH 6–7 by addition of $NaHCO_3$ and loaded onto glass columns containing 1 ml Dowex AG 1-X8 (BioRad, NSW, Australia) for separation by anion exchange chromatography. Inositol phosphates were eluted with ammonium formate as described by Bird [20]. To each 2 ml fraction, scintillation fluid (ICN, NSW, Australia) was added and radioactivity counted in a liquid scintillation counter (Beckman, Australia).

2.7. Assay of protein kinase C activity

The cytosolic and particulate fraction of cardiac myocytes were obtained before and after stimulation using the method of Harnett [21] and protein kinase C activity assayed in the fractions using a commercially available Protein kinase C enzyme assay system (Amersham, Australia).

Protein content in both the fractions was determined using the modified Bradford method [22]. PKC activity was measured as the increase in ^{32}P incorporation in the absence (basal) or presence (stimulated) of α_1- adrenergic stimulation in fatty acid supplemented cells and expressed as a percentage of control cells.

2.8. Statistical analyses

All data are shown as means ± SEM. The results of fatty acid supplementation on IP_3 and IP_4 release and PKC activity were analyzed using analysis of variance (ANOVA) using Statview 4.5 and Fisher's Exact Test.

3. Results

3.1. Fatty acid composition of PtdIns and DAG

To understand the effect of n-3 PUFA on phospholipid fatty acid composition, the fatty acid composition of PtdIns reported in our earlier work [13] has been presented (Table 1). In that study after 24 hr incubations with the AA, EPA and DHA, incorporation of EPA and DHA in EPA-cells and DHA-cells respectively were highest in the PI fraction (36.9 ± 1.1% EPA and 52.5 ± 1.7% DHA) compared to control-cells ($p < 0.05$). The increase of EPA and DHA in the PI fraction was accompanied with a decrease in the linoleic acid and arachidonic acid content.

In the DAG fraction (Table 2), there was incorporation of EPA and DHA in the respective fatty acid supplemented cells but they were not significantly different from that of the control cells. In the EPA-cells, the incorporation was accompanied by a decrease in the AA content but this was not observed in the DHA-cells.

3.2. Inositol phosphate release after n-3 fatty acid supplementation in cardiac myocytes

When cardiac myocytes supplemented with different fatty acids were stimulated with epinephrine (0.5 mM) and

Table 1
Fatty acid content of the PtdIns fraction in porcine cardiac myocytes incubation with different fatty acids

	Fatty acid content (g/100g)			
	Control	AA	EPA	DHA
C16:0	27.2 ± 2.1[a]	36.4 ± 4.8[b]	24.2 ± 0.4	18.2 ± 0.7[b]
C16:1	3.6 ± 0.5	4.0 ± 0.8	1.7 ± 0.3	2.0 ± 0.3
C18:0	11.2 ± 0.7[a]	6.5 ± 0.2[b]	4.5 ± 0.2[b]	2.9 ± 0.1[b]
C18:ln9	13.5 ± 0.6[a]	6.2 ± 0.7[b]	3.2 ± 0.3[b]	2.5 ± 0.2[b]
C18:ln7	4.5 ± 0.5	3.7 ± 0.1	2.0 ± 0.1	1.8 ± 0.2
C18:2n6	25.3 ± 4.2[a]	23.0 ± 1.0[b]	2.7 ± 0.4[b]	10.2 ± 0.6[b]
C18:3n3	0.2 ± 0.2	0.8 ± 0.1	0.2 ± 0.0	0.4 ± 0.1
C20:2n6	0.6 ± 0.3	0.7 ± 0.4	0.4 ± 0.0	0.4 ± 0.0
C20:4n6	1.8 ± 0.2[a]	1.8 ± 0.5	1.1 ± 0.1	0.6 ± 0.0[b]
C20:5n3	1.4 ± 0.7[a]	1.2 ± 0.9	36.9 ± 1.1[b]	0.1 ± 0.0
C22:5n3	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.0	0.2 ± 0.0
C22:6n3	1.2 ± 1.2[a]	0.1 ± 0.1	0.4 ± 0.0	52.5 ± 1.7[b]

Isolated porcine cardiac myocytes were incubated with 400 μM each, AA, EPA and DHA. After 24 hours, lipid was extracted and phospholipids separated by TLC. The values shown are means ± SEM from three different experiments. Values with different superscripts across individual rows indicate statistically significant difference, $P < 0.05$.

the specific α_1-receptor agonist, phenylephrine (0.5 mM), there was an increase in total inositol phosphates released in all four groups (data not shown). Though there was no difference in IP_3 levels before stimulation, the levels of IP_3 and IP_4, the second messengers of interest in this study, were significantly different ($p < 0.01$) between all three fatty acid groups after α_1-adrenergic stimulation (Figure 1). Cardiac myocytes supplemented with arachidonic acid had the highest increase in IP_3 release compared to control cells (185.9%) followed by cells supplemented with EPA (134.1%) and with DHA (119.4%). A similar trend was observed in the release of IP_4 after stimulation. Arachidonic acid supplemented cells had the highest release compared to control cells (162.4%) while EPA and DHA cells had lower IP_4 levels compared to control cells (90.5% and 64%, respectively).

3.3. Activity of PKC after fatty acid supplementation in cardiac myocytes

Activation of PKC follows the formation of DAG after agonist induced inositol phospholipid hydrolysis at the plasma membrane. In this study α_1-receptor stimulation by epinephrine and phenylephrine in the fatty acid supplemented cardiac myocytes caused the redistribution of cytosolic activity to the particulate fraction (Table 3). EPA supplemented cells had the lowest cytosolic PKC activity in basal (non-stimulated) conditions and after stimulation (30.7 ± 6.9% and 6.9 ± 3.9% of control, respectively). AA and DHA supplemented cells had similar PKC activity in non-stimulated cells but after stimulation, cytosolic activity of DHA supplemented cells was significantly lower (33.3 ± 8.9% compared to 83.4 ± 11.7%) than AA supplemented cells.

Table 2
Fatty acid content of the DAG fraction of porcine cardiac myocytes after incubation with different fatty acids

	Fatty acid content (g/100g)			
	Control	AA	EPA	DHA
C14:0	2.1 ± 0.7	3.6 ± 0.9	2.0 ± 0.4	0.8 ± 0.8
C16:0	24.4 ± 5.0	29.4 ± 1.0	21.7 ± 3.1	16.3 ± 2.4
C18:0	16.4 ± 2.3	16.2 ± 2.0	16.2 ± 3.3	15.3 ± 1.1
C18:ln9	21.0 ± 10.0	14.1 ± 10.2	18.9 ± 7.1	21.6 ± 6.4
C18:ln7	1.2 ± 0.8	1.4 ± 1.4	1.9 ± 1.1	2.1 ± 1.1
C18:2n6	6.4 ± 3.2	3.8 ± 0.8	9.2 ± 4.1	4.7 ± 0.6
C18:3n3	2.6 ± 1.6	1.2 ± 1.0	1.6 ± 0.4	1.2 ± 1.2
C20:ln9	16.8 ± 8.7	12.0 ± 6.1	13.6 ± 2.7	16.5 ± 2.6
C20:4n6	2.6 ± 0.3	2.3 ± 0.7	1.0 ± 0.5	3.3 ± 0.8
C20:5n3	5.0 ± 0.7	3.5 ± 1.2	3.9 ± 0.5	4.5 ± 0.4
C22:0	1.1 ± 1.1	2.4 ± 0.9	2.3 ± 0.7	1.4 ± 0.7
C22:ln9	0.5 ± 0.7	4.6 ± 3.2	2.1 ± 1.1	3.5 ± 3.5
C22:5n3	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.9	1.1 ± 1.1
C22:6n3	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 1.5	1.7 ± 1.3

Isolated porcine cardiac myocytes were incubated with 400 μM each, AA, EPA and DHA. After 24 hours, lipid was extracted and DAG separated by TLC. The values shown are means ± SEM from three different experiments. Only major fatty acids have been presented.

Fig. 1. IP_3 and IP_4 release after α_1-adrenoreceptor stimulation in cardiac myocytes incubated with different fatty acids. Values are the means ± SEM, from two separate experiments (n = 6). Stimulation in basal and stimulated cells have been expressed as a proportion of control cells. *Significantly different to control, $P < 0.05$.

In the particulate fraction, PKC activity was the lowest in AA supplemented cells under basal conditions (42.2 ± 4.1% compared to controls). Though there was no significant difference in particulate PKC activity between AA, EPA and DHA supplemented cells in basal conditions and after stimulation, the pattern of activation of PKC between cells were very different. Cells supplemented with DHA had the highest PKC activity associated with the particulate fraction (580.3 ± 307.2% compared to controls). EPA supplemented cells had the lowest PKC activity (59.2 ± 18.2% compared to controls) after stimulation. The SEM values, especially in the particulate fractions, were high, the reason for which is not clear except for being experimental.

4. Discussion

We previously found that in cardiac myocytes supplemented with EPA and DHA, the composition of the PI fatty acid was modified after supplementation with these fatty acids [13]. The n-6 fatty acid in these cells had been replaced with the n-3 fatty acids and we hypothesized that substitution of n-6 fatty acids by n-3 fatty acids in membrane phospholipids could modify the PLC mediated hydrolysis of inositol phosphates. DAG, the other product of PIP_2 hydrolysis that remains in membranes is believed to initiate the activation of PKC. Although it is known that a highly specific lipid-protein interaction is required for PKC in that only DAG in the sn 1,2 configuration is able to activate PKC, no extensive information is available regarding the importance of fatty acid composition of DAG for enzyme activation. From this study, the data on IP_3 and IP_4 release after α-adrenergic stimulation in EPA and DHA supplemented cells confirm our hypothesis. It was therefore our aim to study if n-3 PUFA supplementation similarly modified 1,2-DAG fatty acids and their subsequent effect on PKC activation.

In this study, although n-3 PUFA increased the levels of these fatty acids in the DAG fraction, they were not significantly different from that of the untreated cells. Though in EPA supplemented cells there was a reduction in AA, in DHA supplemented cells, despite the enrichment, it did not alter the levels of AA. The fatty acid profile of DHA supplemented cells in this study are similar to that obtained by Bordoni et al. [23] in the DAG fraction where no difference in the level of AA in DHA supplemented cells were observed. The difference observed in the pattern of incorporation between EPA and DHA in DAG in our study and the subsequent difference seen in their activation of PKC may be explained by the relative amounts of these fatty acids present in membranes as well as the difference in their metabolism. Agonist induced hydrolysis of other membrane phospholipids, particularly phosphatidylcholine (PC) by phospholipase D (PLD) and A_2 (PLA_2) may also play a part in cell signaling [24]. In addition, the appearance of DAG in membranes is transient and its rapid disappearance could be due to its conversion back to inositol phospholipids by way of phosphatidic acid (PI turnover) and to its further degradation to AA [25].

Hrelia et al [26] found that in rat cardiac myocytes DAG enriched with DHA, after α_1-adrenergic stimulation, particulate PKC activity remained higher than basal values. They

Table 3
Protein Kinase C activity in cytosolic and particulate fractions of cardiac myocytes supplemented with different fatty acids

	Protein Kinase C activity (% Control)					
FA supplemented cells	AA		EPA		DHA	
	Basal	Stimulated	Basal	Stimulated	Basal	Stimulated
Cytosolic	119.2 ± 20.9[a]	83.4 ± 11.7[a]	30.7 ± 7.0[a]	6.9 ± 3.9[b]	119.2 ± 15.9[a]	33.3 ± 8.9[b]
Particulate	48.8 ± 4.1	122.0 ± 21.3	102.1 ± 59.9	59.2 ± 18.2	248.7 ± 90.5	580.3 ± 307.2

Cardiac myocytes were incubated with 400 μM AA, EPA or DHA. After 24 hours, the cells were stimulated with epinephrine and α_1-receptor agonist, and cytosolic and particulate fractions obtained. PKC activity was assayed in the fractions using a PKC enzyme assay system. Values are presented as means ± SEM (n=6). PKC activity is expressed as a proportion of that in control cells.

[a,b,] significantly different, $P < 0.05$.

speculated that the possible reason for the more persistent activation of PKC was due to the DHA containing DAG being a poor substrate for DAG-kinase. In our study, we observed a similar higher particulate PKC activity after stimulation in cells supplemented with DHA.

Responses to activation of PKC in the heart include phosphorylation of the myosin light chain (MLC) protein which causes muscle contraction [27] modulation and activation of ion channels which include the L-type Ca^{2+} channel, Na^{+} and Cl^{-} channels. Concentration of PKC in the heart is much lower than that found in brain and the amount of PKC relative to protein is believed to decline post-natally in the heart which explains the low activities and difficulties in detecting PKC in adult tissue [28]. The difference in activity of PKC in EPA and DHA supplemented cells in this study, in both the cytosolic and particulate fraction could be a reflection of the difference seen in DAG incorporation. The lower PKC activity observed in EPA supplemented cells, both in the cytosolic as well as particulate fraction could suggest the possibility of an alternate pathway for EPA containing phospholipids. In contrast, using 3T3 fibroblast cell Vernhet et al. [29] showed that EPA- containing DAG are potent in vitro PKC activators and hence did not support the hypothesis of alteration of PKC activity mediated through generation of dietary n-3 PUFA containing DAG. Membrane bound and cytosolic enzymes could have a higher affinity for AA containing DAG, which could explain the higher levels of translocation and higher activity in particulate fraction of AA supplemented cells in this study.

Transition from a hunter-gatherer lifestyle to modern dietary practices has led to dramatic shifts in dietary intakes [30]. n-3 PUFA has been shown to alter the activity of membrane associated adrenergic receptors and thereby modulating the receptor mediated response. α_1-adrenoreceptor activation leads to an increase in intracellular Ca^{2+} concentrations while α_2- and β-adrenoreceptor activation inhibits and stimulates cAMP formation respectively [31]. The results obtained after β blocking in this study is different to that observed in our earlier in vivo study where cardiac myocytes were isolated from pigs fed a diet supplemented with fish oil or beef tallow for 6 weeks (unpublished work). In that study preincubation with propranolol in cardiac myocytes isolated from pigs fed the fish oil diet, further attenuated the release of IP_3 and IP_4 compared to the myocytes isolated from beef tallow supplemented pigs. This observation demonstrates the difference in receptor mediated hydrolysis of inositol phosphates in vitro and in vivo. In a study by Locher et al. [32] increasing membrane content of EPA appeared to block the ability of the vasopressin receptor to activate PLC-β as well as significantly decrease agonist stimulated IP_3 release, suggesting a fish oil mediated suppression of PLC-β activation.

In a study by Woodcock et al. [33] in left atrial myocytes isolated from rats fed (n-3) PUFA and (n-6) PUFA enriched oils for 8 weeks, the total release of inositol phosphates was reduced both with and without stimulation by nor-epinephrine. Karmazyn et al. [34] showed that there was an increase in Ca^{2+} uptake by the endoplasmic reticulum in rats raised on a fish oil enriched diet which could possibly explain the prevention of arrhythmias by n-3 PUFA. Membrane PKC was found to be markedly increased in ischemic hearts along with phosphorylation of endogenous proteins [35]. High fat diets in general appear to increase PKC activation with adverse consequences for both vascular and cancer risk, whereas the long chain n-3 PUFA from fish oil under some circumstances are believed to limit PKC activation [36]. Administration of diets enriched with oleic, linoleic or with EPA and DHA in rabbits resulted in modified production of inositol phosphates in platelets when stimulated [37]. They found that stimulation of IP_3 generation was lower in the (n-3) PUFA-fed animals compared to other groups. Similar inhibitory effect of fish oil administration on IP_3 has also been reported in guinea pig epidermis [38] and in stimulated platelets after in vitro incubation of EPA [39]. The levels of both IP_3 and IP_4 were significantly reduced after α_1-adrenergic stimulation in this study, which confirms these earlier reports.

In this study we did not investigate if any eicosanoids were produced after n-3 and n-6 fatty acid supplementation. There are reports to suggest that eicosanoids might indirectly activate PLC while arachidonic acid metabolites produced via the cytochrome P450 pathway can cause biological effects via activation of PKC [40].

From these results, it is evident that n-3 PUFA supplementation modifies membrane phospholipid fatty acid composition and modulates the second messenger signaling pathways in the adult cardiac myocytes. More detailed studies are required to determine the effect of PKC activation on protein phosphorylation as well as calcium channels within cardiac myocytes. Studies investigating intracellular calcium release in cardiac myocytes after fatty acid supplementation are currently being undertaken in our lab.

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