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Dietary long-chain *n*-3 fatty acids modify blood and cardiac phospholipids and reduce protein kinase-C-δ and protein kinase-C-ϵ translocation

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The effects of an *n*-3 PUFA-enriched diet on cardiac cell membrane phospholipid fraction compositions and associated protein kinase-C (PKC) translocation modification have never been studied in higher mammals. This is of importance since membrane fatty acid composition has been shown to influence PKC signalling pathways. In the present study, we have tested whether the incorporation of *n*-3 PUFA in cardiac membrane phospholipids correlated with changes in the fatty acid composition of diacylglycerols (DAG) and led to a differential translocation of PKC isoforms. Two groups of five dogs were fed the standard diet supplemented with palm oil or fish oil for 8 weeks. Dogs fed a fish oil-enriched diet showed a preferential incorporation of EPA and, to a lesser extent, of DHA, at the expense of arachidonic acid, in the circulating TAG, plasma phospholipids, erythrocyte phospholipids and cardiomyocyte phospholipid fractions. Analysis of 1,2-DAG fatty acid composition also indicated a preferential enrichment of EPA compared with DHA. Associated with these results, a reduction in the expression of PKC-δ and PKC-ϵ isoforms in the particulate fractions was observed whereas no effect was seen for PKC-α and PKC-ζ. We conclude that a fish oil-enriched diet induces a modification in fatty acid composition of cardiac membrane phospholipids, associated with a differential translocation of PKC isoforms. These results can be explained by the production of structurally different DAG that may participate in some of the protective effects of *n*-3 PUFA against various chronic diseases.

Long-chain *n*-3 fatty acids: Fish oils: Cardiac membrane phospholipids: Protein kinase-C

The mammalian protein kinase-C (PKC) family comprises at least ten isozymes divided into three subclasses based on their structures and their mode of activation: (1) the conventional PKC, consisting of the α, βI, βII and γ isozymes, are activated by Ca^{2+} and diacylglycerol (DAG) in the presence of phosphatidylserine; (2) the novel PKC isozymes, consisting of the δ, ϵ, η and θ isozymes, do not require Ca^{2+} for activation; (3) atypical PKC isozymes, consisting of the ζ and ι/λ isozymes, are Ca^{2+} independent and are not stimulated by DAG or phorbol esters[1,2]. During an α_1-adrenergic stimulation, some PKC isoforms are activated, leading to their translocation from the cytosolic to the plasma membrane fraction. The implicated isoforms were shown to be PKC-δ[3], or PKC-ϵ and -δ[4,5] or PKC-ϵ and -α[6].

n-3 Fatty acids are known to modulate PKC activities[7,8]. In particular, the PUFA at the sn-2 position of DAG selectively affect the activity of DAG-activated PKC[9,10].

Kim *et al.*[8] have shown that EPA is less efficient than DHA at inhibiting the catalytic subunit of PKC-β of rat brain *in vitro*. Fan *et al.*[11] found that dietary DHA suppressed the PKC-θ signalling pathway of mouse T-lymphocytes following a modification of raft composition. Such an inhibition has been proposed to explain the anti-inflammatory effects of *n*-3 PUFA. It was also observed that Jurkat cells quickly incorporate EPA and DHA (after approximately 2 h) with an efficiency varying with the phospholipid fraction (phosphoinositides (PI) = phosphatidylserine > phosphatidylethanolamine (PE) > phosphatidylcholine (PC)). This incorporation was associated with a reduction of PKC-α and PKC-ϵ translocation with no effect on PKC-δ[12]. However, the majority of studies have been performed *in vitro*. Thus, to evaluate the effects of nutritional intake and take into account differences in PUFA metabolism, it is of importance to work *in vivo* with animal models. Such experiments are complementary of *in vitro* experiments.

The purpose of the present investigation was to determine, in dogs, the effects of a dietary palm oil and fish oil supplementation on different plasma lipid fractions, i.e. NEFA,

Abbreviations: AA, arachidonic acid; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphoinositide; PKC, protein kinase-C; Tris, tri(hydroxymethyl)-aminomethane.

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TAG and phospholipids, on erythrocyte phospholipids and cardiomyocyte phospholipids and DAG. We compared the influence of these two different diets on the translocation of four PKC isozymes: α, δ, ϵ and ζ. Our hypothesis was that EPA and DHA may affect, in part, the PKC signalling pathway and, consequently, influence cellular physiology in health and disease.

Methods

Chemical and reagents

PKC isozyme-specific antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibody goat anti-rabbit IgG were obtained from TEBU (Le Perray en Yvelines, France). Chemical reagents, enhanced chemiluminescent (ECL) Western blotting reagents and ready-to-use gels (NuPage™ 10 % Bis-Tris gel) were obtained from InVitrogen (Cergy Pontoise Cedex, France) and high-performance luminescence detection film (Hyperfilm-ECL) was from Amersham (Les Ulis, France). All other reagents were from Sigma-Aldrich (Lyon, France).

Dogs

Ten healthy male adult Beagles (median body weight, 15·85 (range 13·85–18·65) kg; median age, 12 (range 9–15) months) were fed a maintenance diet (UAR 125 C3; SAFE, Villemoisson, Epinay-sur-Orge, France) for 2 weeks before the study. Dogs were housed and cared for at CERB (Baugy, France). Each dog was housed separately. Dogs were provided light for 12 h per d (08.30 to 20.30 hours).

The study plan relating to the present study was reviewed by the CERB internal ethics committee and complies with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (publication no. 85–23, revised 1996).

Experimental procedures

Dogs were randomly assigned to one of the following diet groups: palm oil group and fish oil group. The palm oil diet reflects a control SFA- and MUFA-enriched diet. As a basal diet, all dogs received UAR 125 C3 feed (SAFE) distributed daily at a fixed time. The daily ration was limited to 400 g during the experiment. The approximate composition of the basal diet (UAR 125 C3) was: protein, 24 %, crude fat, 2·4 % (in mol%: 16: 0, 16·6; 18: 0, 8·5; 16: 1, 3·1; 18: 1, 38·1; 18: 2*n*-6, 33·7); parietable glucids (cellulose), 3 %. The palm oil group dogs (*n* 5) had their diet supplemented with palm oil (600 mg/kg per d) administrated in non-gastro-resistant gelatine capsules while the fish oil group dogs (*n* 5) were supplemented with fish oil in the same way (see Table 1). At the end of the 8 weeks of dietary supplementation, the dogs weighed 17·5 (SEM 1·0) kg in the palm oil group and 16·6 (SEM 0·9) kg in the fish oil group.

Blood samples (10 ml), obtained from the lateral cephalic vein, were collected once per week before the ration was given. Blood was drawn into EDTA-containing tubes and was kept on ice until plasma preparation. Plasma and erythrocytes were prepared within 30 min of sampling by centrifugation for 10 min at 3000 rpm (+4 ± 2°C). Plasma was divided into two propylene tubes (2 ml per tube) which were closed under N_2 and immediately frozen in liquid N_2, then stored at −80 ± 10°C until analysis. Erythrocytes were stored using the same protocol.

On the day of euthanasia, animals were anaesthetised by intravenous injection of pentobarbital (30 mg/kg in a volume of 1 ml/kg). The animal was placed in a lateral recumbency on a necropsy table. The heart was quickly removed and placed in an oxygenated and heparinised (about 1 U/ml) Tyrode's solution maintained at +4°C (composition in mM: NaCl, 118; KCl, 4; $NaHCO_3$, 27; $MgCl_2$, 1; NaH_2PO_4, 1·8; $CaCl_2$, 1·8; glucose, 11; pH, 7·35 − 7·45). Pieces of left ventricle (approximately 1 cm^3) were quickly collected, then frozen in liquid N_2 and stored frozen (−80 ± 10°C) until analysis. The overall time between the excision of the heart and the freezing of cardiac pieces never exceeded 5 min. In such conditions, the content of 1,2-DAG is unlikely to be affected[13].

Quantitative fatty acid analysis

Lipids were extracted from the plasma according to Haan *et al.*[14] and Detmer *et al.*[15] with chloroform–methanol–acidified distilled water (pH 2) (2·4:1:1, by vol.). After centrifugation, the lower phase containing lipids was collected and the aqueous phase was re-extracted with chloroform–methanol (2:1, v/v) and centrifuged. The lower organic phases were dried under N_2. Samples were taken for chromatographic separation of NEFA, TAG and phospholipid fractions.

Lipids were extracted from erythrocytes according to the method previously described by Rose & Oklander[16]. Briefly, 1 ml erythrocytes was mixed with 1 ml distilled water, 11 ml isopropanol and 7 ml chloroform. After centrifugation, the supernatant fraction corresponding to the organic phase was collected and filtered on sodium sulfate. The solvent was evaporated and lipids were dissolved to a concentration of 10 mg/ml with chloroform. Samples were taken for chromatographic separation of the total phospholipids fraction.

Lipids were extracted from the cardiac tissues by the method of Folch *et al.*[17]. The heart samples were minced, rinsed in ice-cold saline to remove contaminating blood, weighed and homogenised with an Ultra Turrax™ T25 (IKA® Works, Inc., Wilmington, NC, USA) in a volume of chloroform–methanol mixture (2:1, v/v). Samples were centrifuged. The pellet was re-extracted with chloroform–methanol (2:1, v/v) and the supernatant fraction (organic phase) was washed with 0·25 volumes of 0·73 % NaCl and centrifuged, leading to a lower organic phase and upper aqueous phase. The lower organic phase was collected and filtered on glass wool and sodium sulfate. The solvent was evaporated and lipids were dissolved to a concentration of 10 mg/ml with chloroform. Samples were taken for chromatographic separation of the phospholipids fraction.

Separation of lipid classes was carried out on TLC plates (silica gel; Whatman PLC, Brentford, Middlesex, UK) using a solvent system comprising hexane–diethyl ether–glacial acetic acid (70:30:1, by vol.) for the separation of plasma and erythrocyte lipid fractions and using 1·8 % boric acid in a solvent system of methanol–chloroform–petroleum

Table 1. Fatty acid composition of the oils used in the experiments*
(Mean values with their standard errors)

	PO day 1		PO day 21		FO day 1		FO day 21	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Fatty acids (mol%)								
14 : 0	1·42	0·02	1·40	0·01	0·02	0·01	0·03	0·00
15 : 0	0·05	0·00	0·06	0·01	0·62	0·05	0·62	0·06
16 : 0	47·03	0·40	46·18	0·07	0·09	0·00	0·10	0·02
17 : 0	0·08	0·01	0·08	0·00	0·27	0·06	0·24	0·03
18 : 0	3·76	0·02	3·70	0·02	2·19	0·02	2·21	0·00
20 : 0	0·27	0·02	0·25	0·02	0·00	0·00	0·00	0·00
22 : 0	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
14 : 1	0·00	0·00	0·00	0·00	0·01	0·00	0·01	0·00
15 : 1	0·02	0·02	0·00	0·00	0·52	0·01	0·50	0·02
16 : 1	0·36	0·10	0·42	0·22	0·04	0·01	0·04	0·01
17 : 1	0·02	0·00	0·01	0·01	4·25	0·05	4·37	0·06
18 : 1	35·38	0·16	35·49	0·21	0·99	0·07	1·03	0·05
20 : 1	0·13	0·01	0·07	0·04	1·80	0·04	1·81	0·05
22 : 1	0·02	0·02	0·00	0·00	0·00	0·00	0·00	0·00
24 : 1	0·02	0·01	0·00	0·00	0·00	0·00	0·00	0·00
18 : 2*n*-6	0·00	0·00	0·00	0·00	0·86	0·04	0·87	0·03
18 : 3*n*-6	9·70	0·14	9·58	0·17	0·11	0·01	0·11	0·02
20 : 2*n*-6	0·09	0·01	0·05	0·00	0·51	0·07	0·50	0·01
20 : 3*n*-6	0·79	0·00	0·96	0·16	0·27	0·00	0·19	0·07
20 : 4*n*-6	0·03	0·02	0·05	0·01	1·35	0·07	1·34	0·04
22 : 2*n*-6	0·01	0·00	0·00	0·00	0·03	0·01	0·04	0·02
22 : 4*n*-6	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
22 : 5*n*-6	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00
18 : 3*n*-3	0·00	0·00	0·00	0·00	0·96	0·02	0·96	0·01
20 : 3*n*-3	0·00	0·00	0·00	0·00	0·34	0·02	0·36	0·01
20 : 5*n*-3	0·00	0·00	0·00	0·00	27·94	0·29	27·71	0·21
22 : 5*n*-3	0·01	0·00	0·01	0·01	4·53	0·15	4·61	0·15
22 : 6*n*-3	0·00	0·00	0·00	0·00	48·46	0·08	48·19	0·54
Total SFA	52·61	0·07	51·66	0·02	3··18	0·02	3·19	0·02
Total MUFA	35·95	0·04	35·98	0·06	7·60	0·02	7·76	0·02
Total *n*-6	10·62	0·02	10·64	0·04	3·12	0·02	3·05	0·02
Total *n*-3	0·01	0·00	0·01	0·00	82·24	0·11	81·84	0·18
Identified	99·19	0·03	98·29	0·03	96·14	0·04	95·84	0·05
Unidentified	0·81	0·03	1·71	0·03	3·86	0·04	4·16	0·05

PO, palm oil; FO, fish oil.
* Lipid pills were prepared at day 1 and their composition was checked at day 1 and day 21 to evaluate possible changes due to lipid peroxidation.

ether–glacial acetic acid (20:40:30:10, by vol.) for the separation of cardiomyocyte phospholipid fractions. After spraying the plates with 2′,7′-dichlorofluorescein (0·2 %, w/v) in ethanol for identification, phospholipid fractions were scraped from the plates. The fractions were transmethylated using BF_3 (14 %, w/v) in methanol and analysed by GC.

Separation of 1,2-DAG was performed by TLC on boric acid-containing silica gel plates. Plates were prepared by soaking in a solution of 2·3 % boric acid in ethanol and were activated by drying for 10 min at 100°C. After application of the lipid samples, prepared in a chloroform–methanol mixture (2:1, v/v), separation of the lipids was performed with a running solvent of chloroform–acetone (96:4, v/v). As for phospholipids, spraying the plates with 2′,7′-dichlorofluorescein (0·2 %, w/v) in ethanol was used for identification and 1,2-DAG fractions were scraped from the plates. Spots of 1,2-DAG were identified on the silica gel plate using 1,2-dioleinglycerol and 1,3-dioleinglycerol as standards (Supelco Sigma-Aldrich, Saint Quentin Fallavier, France). The fractions were transmethylated using BF_3 (14 %, w/v) in methanol and analysed by GC.

Gas chromatography analysis

The analysis by GC was performed on a GC Trace apparatus (Thermo Finnigan, Les Ulis, France) equipped with an on-column injector, a flame ionisation detector and a BPX70 polar capillary column (50 m, 0·32 mm internal diameter, 0·25 μm film thickness; SGE France, Villeneuve St. Georges, France). Fatty acid methyl ester peaks were identified by comparing their retention times with those of a commercially available mixture of fatty acid methyl esters (Supelco 37 components FAME; Supelco Sigma-Aldrich).

Western blots

Frozen pieces of left ventricle (about 1 cm^3) were minced and homogenised with an Ultra Turrax™ T25 (IKA® Works, Inc., Wilmington, NC, USA) in 10 ml of a buffer containing (mmol/l): tri(hydroxymethyl)-aminomethane (Tris)-HCl, 20 (pH 7·5); sucrose, 250; EDTA, 2; ethylene glycol tetra-acetic acid, 2; 2-mercaptoethanol, 10; dithiotreitol, 10. The buffer was supplemented with protease inhibitors PMSF (1 mmol/l)

and leupeptin (25 mmol/l). The homogenate was centrifuged at 100 000 **g** for 75 min at +4°C. The supernatant fraction was removed and considered as the soluble fraction. The pellet was re-suspended in the homogenisation buffer containing 0·5 % Triton X-100, incubated for 30 min at 4°C, then centrifuged at 25 000 **g** at +4°C. The supernatant fraction was considered as the particulate extract. Protein content determination was performed with the bicinchoninic acid (BCA) assay (Uptima® UP 40 840; Interchim, Montiuçon, France). Protein homogenates were diluted 1:1 with 4 × lithium dodecyl sulfate sample buffer (NuPAGE® LDS sample buffer; InVitrogen, Cergy Pontoise Cedex, France). Equal amounts of proteins were loaded in each lane. Proteins were separated by electrophoresis and transferred from the gel to a polyvinylidene xuoride (PVDF) membrane (Immobilon™-P Transfer Membrane; Millipore Corp., Bedford, MA, USA) using an electroblotting apparatus (InVitrogen, Cergy Pontoise Cedex, France). Membranes were incubated for 2 h at room temperature in 5 % dry milk, Tris-buffered saline (Tris (20 mmol/l), NaCl (137 mmol/l) and 0·1 % Tween-20, pH adjusted to 8) to block non-specific binding sites. After washing in Tris-buffered saline solution five times (5 min each), membranes were incubated for 90 min with polyclonal antibodies directed against the carboxy terminal extremity of specific PKC isoforms, α, δ, ϵ and ζ, at a dilution of 1:2000. After washing in Tris-buffered saline solution five times (5 min each), membranes were incubated with horseradish peroxidase-labelled anti-rabbit IgG diluted at 1:500 for 1 h at room temperature. The blots were visualised using an enhanced chemiluminescent Western blotting detection kit (Amersham, France).

The amount of PKC isoforms in each fraction was measured by densitometry. PKC content was expressed in arbitrary units according to the integration of the area under the densitometric peak.

Statistics

All data are expressed as mean values with their standard errors of n observations. The results of fatty acid concentrations in blood were analysed using repeated-measures ANOVA with *post hoc* analysis where significance was seen. Analysis focused on changes in variables from day 1 to other days of the experiment. Analysis of fatty acid composition in phospholipids and DAG was performed with a one-way ANOVA followed by a Holm–Sidal test where significance was seen. Regarding the results of fatty acid concentrations in cardiac phospholipids and the particulate:soluble ratios of PKC isoforms in cardiomyocytes, statistical analysis was performed using a one-way ANOVA followed by a Bonferroni or Kruskal–Wallis test, as appropriate. Statistical significance was considered at a P value of $<0{\cdot}05$.

Results

Fatty acid concentrations in the plasma

The fatty acid composition of plasma lipids was assessed as a reflection of the intake of lipids from the diet. During the experiment, the relative concentrations of saturated, monounsaturated and polyunsaturated NEFA did not change in the palm oil group while in the fish oil group a significant increase in *n*-3 NEFA, mainly EPA and DHA, was observed (Fig. 1 (a)). The same observation applies to plasma TAG and phospholipids (Fig. 1 (b) and (c)) but, in these latter cases, EPA was incorporated preferentially to DHA. The incorporation of PUFA reached a steady state 2–3 weeks after the diet started.

Fatty acids in erythrocyte phospholipids

Fatty acids of erythrocyte membrane phospholipids were assessed to monitor the changes in tissue membrane phospholipids during the course of the experiment. Similarly to what was observed in the plasma, erythrocyte phospholipids incorporated EPA preferentially to DHA at the expense of arachidonic acid (AA) (Fig. 2). This incorporation did not reach a clear steady state at the end of the experiment.

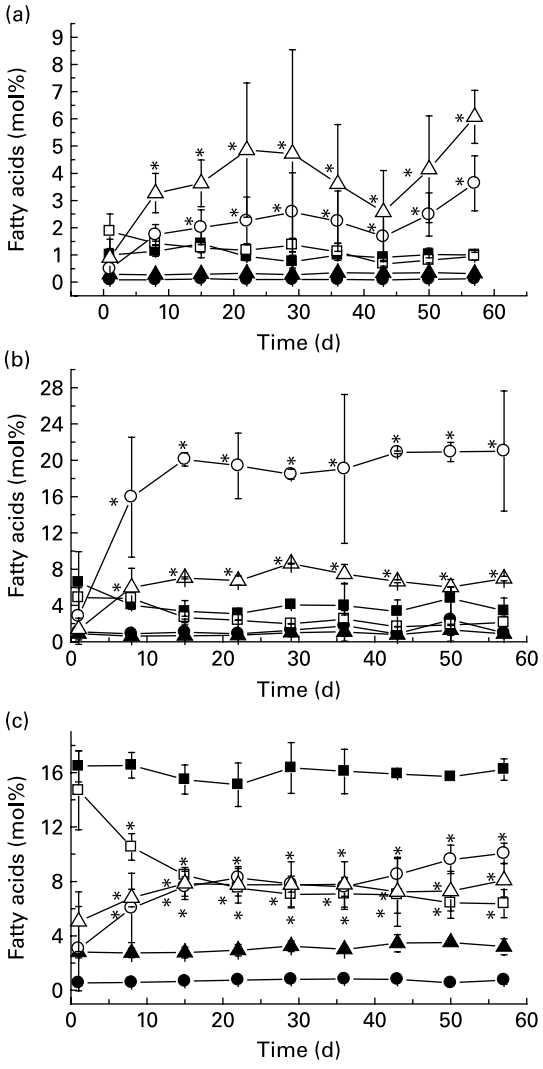

Fig. 1. Evolution of the arachidonic acid (■, □), EPA (●, ○) and DHA (▲, Δ) contents in three lipid fractions of the plasma of dogs supplemented with palm oil (■, ●, ▲) or fish oil (□, ○, Δ) during the experimental diet protocol. The composition was determined in three fractions of the plasma: NEFA (a), TAG (b) and phospholipids (c). Values are means for five dogs, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the dogs fed palm oil (taken as baseline values; $P<0{\cdot}05$).

Fatty acids in cardiac membrane phospholipids

In the palm oil group, AA was the main long-chain PUFA of the different phospholipid fractions, followed by DHA, then EPA (Table 2). The highest levels of these PUFA were observed for PE and PI fractions. Feeding dogs with fish oil led to an important incorporation of *n*-3 PUFA at the expense of AA. This incorporation was more important for EPA than for DHA and, expressed as the ratio EPA:DHA, was more marked in the following sequence of phospholipid fractions: PC > PI = PE > phosphatidylserine. Such changes in phospholipid fatty acid composition might lead to downstream changes in DAG composition after phospholipids were hydrolysed by phospholipases.

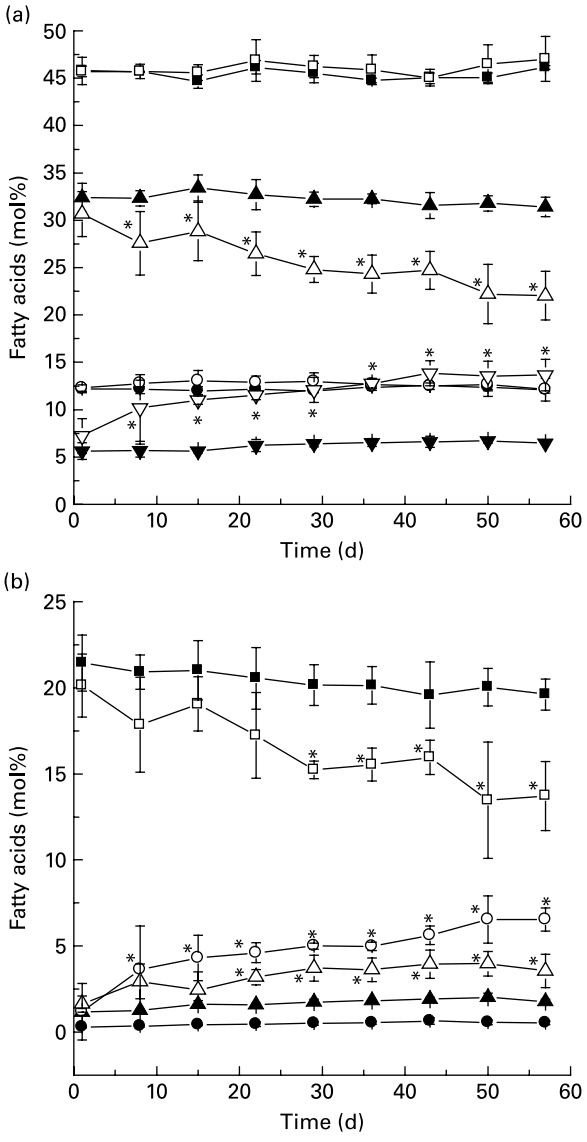

Fig. 2. Fatty acid composition of the erythrocyte phospholipids of dogs supplemented with palm oil (■, ●, ▲, ▼) or fish oil (□, ○, Δ, ▽) during the experimental diet protocol. (a) Evolution with time of the fatty acid classes (SFA (■, □), MUFA(●, ○), and *n*-6 (▲, Δ) and *n*-3 (▼, ▽) fatty acids) incorporated in phospholipids during the diet protocol. (b) Evolution of the arachidonic acid (■, □), EPA (●, ○) and DHA (▲, Δ) contents during the diet protocol. Values are means for five dogs, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the dogs fed palm oil ($P<0{\cdot}05$).

Fatty acids in cardiac membrane diacylglycerols

Similarly to what was observed for cardiac phospholipid fractions, the fish oil diet led to an increase in EPA and DHA in DAG (Table 3) with a preferential enrichment for EPA. The level of EPA and DHA, expressed in mol%, increased significantly from 1·01 (SE 0·3) and 1·48 (SE 0·19) to 8·44 (SE 1·48) and 8·33 (SE 1·15), respectively.

Cardiac protein kinase-C isoform translocation

PKC isoforms were quantified using Western blots as described by Rouet-Benzineb *et al.*[18]. Quantification was performed in the soluble (cytosolic) and particulate (membrane-associated) fractions of each dog separately, and not considering only the diet group. Since translocation of PKC to the membrane leads to its activation, calculating the particulate fraction:soluble fraction ratio gives an index of the degree of activation of the different isoforms of PKC. As shown on Fig. 3, fish oil diet supplementation led to a significantly reduced activation of the ϵ and δ PKC isoforms while the α and ζ isoforms were not affected.

Discussion

The aim of the present study was to analyse whether there was a specific incorporation of *n*-3 PUFA in particular cardiac phospholipid fractions and consequently in cardiac membrane 1,2-DAG. The final goal was to correlate such changes with PKC isoform-specific differential translocation. The main results of the study are that *n*-3 PUFA incorporate preferentially in PE, PC and PI phospholipidic fractions.

The diet we used contained twice as much DHA as EPA. This ratio was reflected in the NEFA composition of the plasma which thus reflects the composition of the diet[19]. However, when esterified in TAG and phospholipids, the ratio was inverted, suggesting a preferential incorporation of EPA *v.* DHA in plasma TAG and phospholipids. Since the blood samples were collected just before feeding, plasma TAG were not carried by chylomicrons but by VLDL which were synthesised by the liver. Two hypotheses can be put forward to explain this surprising result: (1) liver enzymes transferring PUFA on the sn-2 position of glycerol prefer C20 (AA or EPA) to C22 (DHA) PUFA; (2) DHA can be retroconverted to EPA[20]. To answer those questions, a better knowledge of the lipid metabolism of the dog is needed. In any case, the preferential incorporation of EPA in TAG might explain the preferential incorporation of EPA *v.* DHA in erythrocytes and cardiac phospholipids. Such a preferential incorporation has not been found in other studies using rodents[21,22]. This apparent discrepancy can be due to the lipid metabolism in species such as pigs and dogs which is different from that in rodents such as rats and guinea-pigs and that the former are closer to man than are the latter.

The analysis of the fatty acid composition of different fractions of cardiac phospholipids indicates that PE, PC

Table 2. Fatty acid compositions of cardiac phospholipids phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphoinositides (PI) of five dogs fed a diet enriched in palm oil (PO) and five dogs fed a diet enriched with fish oil (FO)

(Mean values with their standard errors)

Phospholipids...	PE				PS				PC				PI			
Diet...	PO		FO		PO		FO		PO		FO		PO		FO	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Fatty acids (mol%)																
14 : 0	0·03	0·00	0·06	0·01	0·08	0·01	0·13*	0·01	0·34	0·04	0·25	0·06	0·27	0·09	0·08	0·02
15 : 0	0·05	0·01	0·07	0·01	0·04	0·01	0·07**	0·01	0·15	0·01	0·24*	0·03	0·08	0·03	0·08	0·03
16 : 0	3·16	0·17	3·93	0·37	3·92	0·47	4·55	0·32	23·69	1·41	20·93	1·68	4·93	0·48	3·34	0·50
17 : 0	0·23	0·02	0·22	0·03	0·24	0·02	0·50**	0·06	0·27	0·03	0·28	0·03	0·52	0·17	0·32	0·02
18 : 0	4·56	0·31	3·27	0·92	43·73	2·02	46·97	2·24	10·21	0·89	11·16	0·97	28·55	1·14	27·75	1·24
20 : 0	0·04	0·00	0·02*	0·00	0·11	0·03	0·11	0·01	0·15	0·06	0·07	0·04	0·07	0·01	0·07	0·01
22 : 0	0·01	0·00	0·04	0·02	0·04	0·01	0·11	0·05	0·03	0·01	0·12**	0·01	0·03	0·01	0·13**	0·01
24 : 0	0·01	0·01	0·00	0·00	0·02	0·00	0·03	0·01	0·02	0·00	0·01*	0·00	0·60	0·25	0·37	0·21
14 : 1	0·04	0·02	0·01	0·00	0·05	0·02	0·03	0·01	0·27	0·09	0·05*	0·02	0·04	0·01	0·04	0·01
15 : 1	0·02	0·01	0·04	0·01	0·03	0·01	0·03	0·00	0·04	0·03	0·02	0·01	0·03	0·02	0·06	0·03
16 : 1	0·39	0·11	0·39	0·08	0·32	0·03	0·37	0·12	0·58	0·09	0·61	0·04	2·36	0·85	1·74	0·93
17 : 1	0·19	0·03	0·16	0·02	0·18	0·06	0·25	0·07	0·27	0·08	0·15	0·01	0·11	0·02	0·15	0·01
18 : 1	32·65	1·13	33·41	1·74	7·00	0·56	4·78**	0·16	25·57	0·67	24·57	1·38	7·32	0·77	6·64	0·32
20 : 1	0·08	0·01	0·08	0·02	0·15	0·04	0·22	0·05	0·04	0·01	0·07*	0·00	0·10	0·01	0·16	0·02
22 : 1	0·00	0·00	0·00	0·00	0·02	0·00	0·02	0·01	0·03	0·01	0·02	0·01	0·08	0·03	0·01*	0·01
24 : 1	1·04	0·05	0·90	0·04	0·43	0·26	0·19	0·08	0·08	0·05	0·32	0·13	0·30	0·18	0·48	0·20
18 : 2*n*-6	6·88	0·68	6·35	0·65	7·52	1·09	6·41	0·75	13·18	0·98	9·50**	0·15	7·25	1·51	3·81	0·31
18 : 3*n*-6	0·02	0·01	0·01	0·01	0·05	0·02	0·05	0·01	0·11	0·00	0·10	0·01	0·06	0·02	0·09	0·02
20 : 2*n*-6	0·22	0·04	0·19	0·01	0·34	0·08	0·29	0·03	0·29	0·09	0·28	0·08	0·27	0·02	0·20	0·03
20 : 3*n*-6	0·40	0·05	0·37	0·05	0·81	0·28	0·32	0·09	0·60	0·13	0·44	0·02	0·55	0·08	0·21**	0·03
20 : 4*n*-6	37·92	0·58	22·57**	0·98	23·98	2·13	15·37**	0·29	11·51	1·65	8·43	0·31	32·35	1·69	21·14**	1·17
22 : 2*n*-6	0·01	0·00	0·01	0·00	0·09	0·01	0·14	0·07	0·07	0·03	0·03	0·01	0·02	0·00	0·01	0·00
22 : 4*n*-6	0·54	0·09	0·20*	0·05	0·53	0·07	0·11**	0·01	0·13	0·03	0·03*	0·01	0·46	0·09	0·17*	0·04
18 : 3*n*-3	0·03	0·00	0·07*	0·01	0·04	0·00	0·07*	0·00	0·07	0·01	0·10*	0·01	0·06	0·01	0·14*	0·03
20 : 3*n*-3	0·10	0·00	0·13	0·01	0·23	0·02	0·11	0·05	0·11	0·03	0·08	0·03	0·28	0·08	0·22	0·06
20 : 5*n*-3	1·10	0·11	12·53**	0·68	0·64	0·16	4·42**	0·35	0·43	0·05	6·44**	0·41	1·19	0·15	13·22**	1·21
22 : 6*n*-3	3·03	0·07	6·82**	0·26	2·11	0·42	2·89	0·25	0·84	0·15	2·70**	0·07	2·61	0·10	6·69**	0·08
Total SFA	8·09	0·33	7·61	0·59	48·19	2·25	52·46	2·42	34·86	2·22	33·06	0·96	35·05	1·33	32·14	1·35
Total MUFA	34·42	1·14	34·99	1·84	8·18	0·88	5·89*	0·20	26·89	0·65	25·80	1·33	10·35	1·38	9·28	0·95
Total *n*-6	46·00	0·99	29·71**	1·31	33·31	1·53	22·68**	0·76	25·89	2·24	18·81*	0·39	40·95	2·37	25·63**	1·53
Total *n*-3	4·26	0·13	19·54**	0·85	3·03	0·55	7·48**	0·53	1·44	0·23	9·33**	0·51	4·15	0·27	20·26**	1·22
Identified	92·77	0·91	91·85	1·47	92·71	1·25	88·52	3·10	89·08	0·52	87·01	2·45	90·50	3·31	87·31	2·33
Unidentified	7·23	0·91	8·15	1·47	7·29	1·25	11·48	3·10	10·92	0·52	12·99	2·45	9·50	3·31	12·69	2·33

Mean value was significantly different from that of the PO group: *$P<0·05$, **$P<0·01$ (one-way ANOVA).

Table 3. Fatty acid compositions of cardiac diacylglycerols of dogs fed a diet enriched in palm oil (PO) or fish oil (FO)

(Mean values with their standard errors)

Diet...	PO		FO	
	Mean	SE	Mean	SE
Fatty acids (mol%)				
14:0	0·38	0·41	0·40	0·01
15:0	0·14	0·04	0·13	0·02
16:0	5·97	3·13	3·99	1·51
17:0	0·17	0·06	0·19	0·01
18:0	7·88	1·70	4·43	0·85
20:0	0·07	0·00	0·10	0·05
22:0	0·05	0·01	0·24**	0·04
24:0	0·00	0·00	0·04	0·06
14:1	0·18	0·01	0·14*	0·01
15:1	0·05	0·02	0·10	0·00
16:1	1·19	0·33	0·41	0·03
17:1	0·18	0·08	0·39	0·35
18:1	17·79	2·56	20·20	2·78
20:1	0·45	0·11	0·61	0·16
22:1*n*-9	0·04	0·03	0·05	0·02
24:1	1·10	0·08	2·42**	0·56
18:2*n*-6	37·04	3·40	31·00	3·44
18:3*n*-6	0·22	0·02	0·04**	0·01
20:2*n*-6	0·57	0·02	0·42	0·12
20:3*n*-6	0·88	0·06	0·45**	0·06
20:4*n*-6	16·35	4·51	9·31*	1·95
22:2*n*-6	0·04	0·01	0·01	0·01
22:4*n*-6	0·78	0·16	0·17**	0·10
18:3*n*-3	0·39	0·03	0·52**	0·01
20:3*n*-3	0·13	0·01	0·10	0·06
20:5*n*-3	1·01	0·30	8·44**	1·48
22:6*n*-3	1·48	0·19	8·33**	1·15
Total SFA	14·65	5·32	9·54	2·37
Total MUFA	20·97	2·40	24·32	2·00
Total *n*-6	55·88	8·14	41·39*	1·78
Total *n*-3	3·01	0·46	17·39**	2·56
Identified	94·51	0·88	92·64	0·42
Unidentified	5·49	0·88	7·36	0·42

Mean value was significantly different from that of the PO group: *$P<0·05$, **$P<0·01$ (one-way ANOVA).

and PI are the most diet-sensitive fractions. PI and PC are major sources of DAG, so changes in their fatty acid compositions can explain the changes in fatty acid composition of 1,2-DAG. However, from the present results, the origin of 1,2-DAG cannot be determined. DAG can be produced either through *de novo* synthesis[23] or through the hydrolysis of PC[24], PI[25] or PE[26]. Production of 1,2-DAG generally displays a biphasic pattern after stimulation[27]; an initial phase of increase where 1,2-DAG is predominantly derived from PI, followed by the late phase of increase where 1,2-DAG is produced from PC[28]. DAG derived from PC, that usually show a more sustained peak than those from PI, have been suggested to lead to a sustained PKC activation[29]. The fatty acid patterns of PC and PI do not change after agonist stimulation and the DAG derived from PC can be distinguished from those derived from PI, based on their fatty acid composition profile[30]. In the present study, 1,2-DAG may be derived from either PI or PC, as judged from the PUFA composition. Therefore, the possibility cannot be excluded that the 1,2-DAG in dog cardiac membranes may be derived from both PI and PC.

Depending on the DHA:EPA ratio of the diet supplementation, differences could be observed in the fatty acid composition of phospholipid and in the activation of PKC isoforms, possibly leading to different physiological effects. We found that translocations of PKC-ζ and PKC-α were not different between the dog groups while translocations of PKC-δ and PKC-ϵ were significantly lower in the fish oil group. Since these latter two PKC isoforms belong to the novel group of PKC which are solely sensitive to DAG, while PKC-α and PKC-ζ belong to the conventional PKC group (PKC-α, sensitive to DAG and intracellular Ca^{2+}) and to the atypical PKC group (PKC-ζ), we propose that the changes in translocation, probably associated with a reduced activation, are linked to the changes observed in phospholipids, more specifically PI and/or PC. However, if so, the reduced translocation of PKC is rather isoform selective. These results are in agreement with those obtained *in vitro* by Madani *et al.*[9] who showed that DAG with EPA or DHA at the sn-2 position were less efficient than those with AA to activate PKC-δ and PKC-ϵ.

It is known that *n*-3 PUFA-enriched food can help to protect individuals against CVD and breast cancer[31,32]. The protection afforded by *n*-3 PUFA is still not understood. One hypothesis involves the PI pathways. Interestingly, such a hypothesis can apply also to diseases such as breast cancer[33]. Indeed, it is known that up regulation of PKC-δ is associated with the development of metastasis (see Debies & Welch[34]). Activation of PKC-ϵ is known to participate in the cardioprotective effects of preconditioning[35] while PKC-δ is involved in the deleterious effects of ischaemia[36]. We observed that both PKC isoforms were less translocated to the particulate fraction in the fish oil diet group but this decrease was more pronounced for PKC-δ than for PKC-ϵ. It is possible that it is not the activity of a given PKC isoform that is important in the cardioprotection but the ratio of the activities of different PKC isoforms. In the case of fish oil feeding, the reduction of PKC-δ translocation is more important, therefore displacing the equilibrium toward the cardioprotective effects afforded by PKC-ϵ.

In conclusion, we observed that feeding dogs with an *n*-3 fatty acid-enriched diet, compared with an SFA- and MUFA-enriched diet, induced modifications of lipid compositions in plasma TAG, phospholipids, erythrocyte phospholipids, cardiac phospholipids and DAG. EPA increased more rapidly and comprehensively in these lipids than DHA. This preferential incorporation was clearly seen in the cardiac PI and PC fractions which are the main sources of DAG in cardiac cells. Associated with these changes, reduced membrane translocations of the PKC-δ and PKC-ϵ isoforms were observed while PKC-ζ and PKC-α translocations were not different. We suggest that these changes in isoform-specific translocation participate in the healthy effects of a fish oil-supplemented diet.

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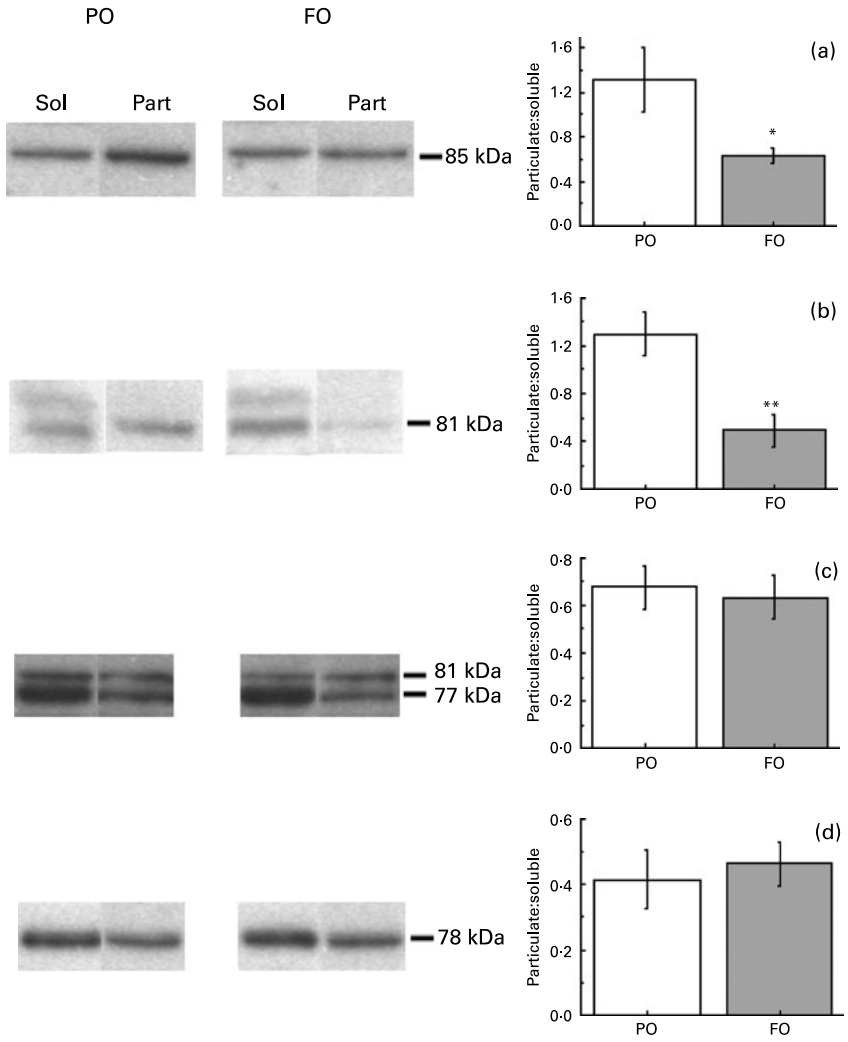

Fig. 3. Western blot analysis of (a) protein kinase-C (PKC)-ε, (b) PKC-δ, (c) PKC-ζ and (d) PKC-α in the soluble (Sol) and particulate (Part) fractions of left ventricular cardiomyocytes. Values are means for five dogs in each diet group, with their standard errors represented by vertical bars. PO, palm oil; FO, fish oil. The graphs represent the particulate:soluble ratio as an index of the basal activity of the different PKC isoforms in cardiomyocytes. Mean value was significantly different from that of the dogs fed palm oil: $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$.

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References

1. Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* **9**, 484–496.
2. Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* **101**, 2353–2364.
3. Puceat M, Hilal-Dandan R, Strulovici B, Brunton LL & Brown JH (1994) Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J Biol Chem* **269**, 16938–16944.
4. Rohde S, Sabri A, Kamasamudran R & Steinberg SF (2000) The α(1)-adrenoceptor subtype- and protein kinase C isoform-dependence of norepinephrine's actions in cardiomyocytes. *J Mol Cell Cardiol* **32**, 1193–1209.
5. Wang L, Rolfe M & Proud CG (2003) Ca(2+)-independent protein kinase C activity is required for α1-adrenergic-receptor-mediated regulation of ribosomal protein S6 kinases in adult cardiomyocytes. *Biochem J* **373**, 603–611.
6. Korzick DH, Holiman DA, Boluyt MO, Laughlin MH & Lakatta EG (2001) Diminished α1-adrenergic-mediated contraction and translocation of PKC in senescent rat heart. *Am J Physiol Heart Circ Physiol* **281**, H581–H589.
7. Lanson M, Besson P & Bougnoux P (1997) Supplementation of MCF-7 cells with essential fatty acids induces the activation of protein kinase C in response to IGF-1. *J Lipid Mediat Cell Signal* **16**, 189–197.
8. Seung Kim HF, Weeber EJ, Sweatt JD, Stoll AL & Marangell LB (2001) Inhibitory effects of ω-3 fatty acids on protein kinase C activity *in vitro*. *Mol Psychiatry* **6**, 246–248.
9. Madani S, Hichami A, Legrand A, Belleville J & Khan NA (2001) Implication of acyl chain of diacylglycerols in activation of different isoforms of protein kinase C. *FASEB J* **15**, 2595–2601.
10. Hichami A, Morin C, Rousseau E & Khan NA (2005) Diacylglycerol-containing docosahexaenoic acid in acyl chain modulates airway smooth muscle tone. *Am J Respir Cell Mol Biol* **33**, 378–386.
11. Fan YY, Ly LH, Barhoumi R, McMurray DN & Chapkin RS (2004) Dietary docosahexaenoic acid suppresses T cell protein

kinase C θ lipid raft recruitment and IL-2 production. *J Immunol* **173**, 6151–6160.
12. Denys A, Hichami A & Khan NA (2005) *n*-3 PUFAs modulate T-cell activation *via* protein kinase C-α and -ε and the NF-κB signaling pathway. *J Lipid Res* **46**, 752–758.
13. Kurz T, Schneider I, Tolg R & Richardt G (1999) α 1-Adrenergic receptor-mediated increase in the mass of phosphatidic acid and 1,2-diacylglycerol in ischemic rat heart. *Cardiovasc Res* **42**, 48–56.
14. Haan GJ, van der Heide S & Wolthers BG (1979) Analysis of fatty acids from human lipids by gas chromatography. *J Chromatogr* **162**, 261–271.
15. Detmer A, Carter AM & Thomas CR (1992) The metabolism of free fatty acids and triacylglycerols by the fetal liver in a guinea pig model of intrauterine growth retardation. *Pediatr Res* **32**, 441–446.
16. Rose HG & Oklander M (1965) Improved procedure for the extraction of lipids from human erythrocytes. *J Lipid Res* **6**, 428–431.
17. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497–509.
18. Rouet-Benzineb P, Mohammadi K, Perennec J, Poyard M, Bouanani Nel H & Crozatier B (1996) Protein kinase C isoform expression in normal and failing rabbit hearts. *Circ Res* **79**, 153–161.
19. Hansen RA, Ogilvie GK, Davenport DJ, Gross KL, Walton JA, Richardson KL, Mallinckrodt CH, Hand MS & Fettman MJ (1998) Duration of effects of dietary fish oil supplementation on serum eicosapentaenoic acid and docosahexaenoic acid concentrations in dogs. *Am J Vet Res* **59**, 864–868.
20. von Schacky C & Weber PC (1985) Metabolism and effects on platelet function of the purified eicosapentaenoic and docosahexaenoic acids in humans. *J Clin Invest* **76**, 2446–2450.
21. Murphy MG, Wright V, Ackman RG & Horackova M (1997) Diets enriched in menhaden fish oil, seal oil, or shark liver oil have distinct effects on the lipid and fatty-acid composition of guinea pig heart. *Mol Cell Biochem* **177**, 257–269.
22. Rousseau D, Helies-Toussaint C, Moreau D, Raederstorff D & Grynberg A (2003) Dietary *n*-3 PUFAs affect the blood pressure rise and cardiac impairments in a hyperinsulinemia rat model *in vivo*. *Am J Physiol Heart Circ Physiol* **285**, H1294–H1302.
23. Peter-Riesch B, Fathi M, Schlegel W & Wollheim CB (1988) Glucose and carbachol generate 1,2-diacylglycerols by different mechanisms in pancreatic islets. *J Clin Invest* **81**, 1154–1161.
24. Exton JH (1990) Signaling through phosphatidylcholine breakdown. *J Biol Chem* **265**, 1–4.
25. Berridge MJ (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu Rev Biochem* **56**, 159–193.
26. Kiss Z & Anderson WB (1990) ATP stimulates the hydrolysis of phosphatidylethanolamine in NIH 3T3 cells. Potentiating effects of guanosine triphosphates and sphingosine. *J Biol Chem* **265**, 7345–7350.
27. Griendling KK, Rittenhouse SE, Brock TA, Ekstein LS, Gimbrone MA Jr & Alexander RW (1986) Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. *J Biol Chem* **261**, 5901–5906.
28. Wright TM, Rangan LA, Shin HS & Raben DM (1988) Kinetic analysis of 1,2-diacylglycerol mass levels in cultured fibroblasts. Comparison of stimulation by α-thrombin and epidermal growth factor. *J Biol Chem* **263**, 9374–9380.
29. Eskildsen-Helmond YE, Hahnel D, Reinhardt U, Dekkers DH, Engelmann B & Lamers JM (1998) Phospholipid source and molecular species composition of 1,2-diacylglycerol in agonist-stimulated rat cardiomyocytes. *Cardiovasc Res* **40**, 182–190.
30. Lamers JM, De Jonge HW, Panagia V & Van Heugten HA (1993) Receptor-mediated signalling pathways acting through hydrolysis of membrane phospholipids in cardiomyocytes. *Cardioscience* **4**, 121–131.
31. Gudbjarnason S & Hallgrimsson J (1976) The role of myocardial membrane lipids in the development of cardiac necrosis. *Acta Med Scand Suppl* **587**, 17–27.
32. Bougnoux P (1999) *n*-3 Polyunsaturated fatty acids and cancer. *Curr Opin Clin Nutr Metab Care* **2**, 121–126.
33. Judé S, Roger S, Martel E, Besson P, Richard S, Bougnoux P, Champeroux P & Le Guennec JY (2006) Dietary long-chain ω-3 fatty acids of marine origin: a comparison of their protective effects on coronary heart disease and breast cancers. *Prog Biophys Mol Biol* **90**, 299–325.
34. Debies MT & Welch DR (2001) Genetic basis of human breast cancer metastasis. *J Mammary Gland Biol Neoplasia* **6**, 441–451.
35. Inagaki K, Hahn HS, Dorn GW II & Mochly-Rosen D (2003) Additive protection of the ischemic heart *ex vivo* by combined treatment with δ-protein kinase C inhibitor and ε-protein kinase C activator. *Circulation* **108**, 869–875.
36. Chen L, Hahn H, Wu G, *et al.* (2001) Opposing cardioprotective actions and parallel hypertrophic effects of δ PKC and ε PKC. *Proc Natl Acad Sci USA* **98**, 11114–11119.

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