(n-3) Long-Chain Polyunsaturated Fatty Acids Prolong Survival following Myocardial Infarction in Rats

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ABSTRACT Many clinical studies report that (n-3) PUFAs decrease the incidence of sudden death in patients with coronary artery disease after myocardial infarction (MI). However, the mechanisms for the beneficial effects of (n-3) PUFAs are unknown. The objectives of the present study were to confirm the findings from clinical trials using an animal model of MI in which dietary intake could be closely controlled and to utilize the model to investigate molecular mechanisms for the beneficial effects of (n-3) PUFAs. Male rats were subjected to coronary ligation to induce MI and were randomly assigned to diets high in (n-6) (58% of lipid) or (n-3) (28% of lipid) PUFAs for 6 mo. A diet high in (n-3) PUFAs was associated with an improvement in 6-mo survival (89.2% vs. 64.9%, \(P = 0.013\)) compared with rats consuming a diet high in (n-6) PUFAs (n = 37/group). In a separate study (n = 5 rats/diet group), the (n-3) PUFA diet decreased the (n-6):(n-3) PUFA ratio in plasma (0.6 ± 0.1 vs. 7.9 ± 1.8, \(P < 0.05\)) and cardiac tissue (0.9 ± 0.1 vs. 11.8 ± 1.6, \(P < 0.05\)) of rats fed for 4 wk. The increased survival in the (n-3) diet group was associated with decreased cardiac activities of protein kinase A and calcium calmodulin–dependent kinase II by 33–38% \((P < 0.05)\) and a 28% decrease \((P < 0.05)\) in phosphorylation (activation) of the ryanodine receptor calcium release channel. Based upon our results, we speculate that decreased activities of protein kinases induced by diets high in (n-3) PUFAs are associated with a decrease in sudden death after MI in rats.


KEY WORDS: infarction sudden death protein kinases lipid metabolism lipid signaling

An estimated 1,100,000 Americans suffer a myocardial infarction (MI) each year, and MI is responsible for one-half of all cardiovascular deaths. An estimated 300,000 individuals suffer sudden cardiac death each year. Although diet clearly modulates the development of atherosclerosis and coronary artery disease, the effect of diet on cardiac arrhythmogenesis is less clear.

Recent studies with (n-3) PUFAs suggest that these agents may decrease the incidence of cardiovascular events and sudden death in patients with ischemic heart disease. Some epidemiologic (1–3) and prospective randomized clinical trials (4–8) reported a decrease in morbidity and mortality from coronary artery disease in patients when diets contain or are supplemented with (n-3) PUFA. In contrast, other epidemiologic (9–11) and randomized controlled trials (12–15) failed to confirm beneficial cardiovascular effects of (n-3) PUFAs. Results from meta-analysis/systematic reviews of (n-3) PUFAs in patients with cardiovascular disease also reported discordant results, with (n-3) PUFA intake reported to either decrease (16,17) or have no effects (18) on mortality. Different levels of dietary (n-3) PUFA intake in different populations of patients may explain some of the discordant results.

Thus, additional studies are warranted to confirm and further define the cardiovascular benefits of (n-3) PUFAs. Mechanistic studies are also required. Understanding the mechanisms by which (n-3) PUFAs modulate the development and progression of cardiovascular disease may improve our understanding of the biochemical basis of the disease and lead to new therapeutic strategies with which to prevent and treat cardiovascular disease.

The goal of this study was multifaceted. We wished to confirm the survival benefits of (n-3) PUFAs in subjects after MI in a tightly controlled environment. Thus, we used an animal model with a genetically homogeneous group of animals and strict dietary control. Secondary goals were to assess the effect of lipid on cardiac lipid content and to probe a number of signaling pathways that are involved in arrhythmia generation. We chose to study protein kinase signaling pathways because these
pathways are linked to the generation of cardiac arrhythmias (19–21). We also wished to establish an animal model responsive to (n-3) PUFAs that would allow for future studies of cellular mechanisms for (n-3) PUFA effects. In this study, we tested the hypothesis that dietary (n-3) PUFA content modulates outcome and protein kinase activities after experimental MI.

MATERIALS AND METHODS

Animals. The studies were performed using 10-wk-old male Wistar rats weighing 250–300 g (Harlan). This study was approved by Methodist Research Institute’s Animal Research Committee and conforms with the NIH guidelines.

Diet. Rats consumed 1 of 2 diets (Research Diets) that differed in the concentrations of (n-6) and (n-3) PUFAs (Table 1). Rats consumed food and water ad libitum. In previous studies in our laboratory, we found that both diets supported similar growth of rats.

Cardiac and plasma lipid analysis. Wistar rats (N = 5 per group) were fed the (n-6) and (n-3) diets for 4 wk, and their hearts and blood were removed. The hearts were frozen in liquid nitrogen and stored at −80°C. The blood was used to isolate plasma and stored at −20°C. Lipids were extracted and analyzed as previously described (22–26). FAME were analyzed on a Shimadzu GC2010 gas chromatograph equipped with an automatic sample injector, flame ionization detector, and a 0.25 mm × 30 m Stabilwax capillary column (Resteck). Tissue fatty acids were identified and quantified against authentic standard FAME (Nu-Chek Prep).

Coronary artery ligation. In a separate study, coronary artery ligation (27,28) was performed using 10-wk-old Wistar rats weighing 250–300 g. Rats were anesthetized using isoflurane (1.5–2%), intubated, and placed on a mechanical ventilator. A left thoracotomy was performed, the pericardium was incised, and the heart was isolated. The left anterior descending coronary artery was ligated using 6-0 prolene sutures 1 mm distal to the take-off of the left main coronary artery. The chest was closed in 2 layers (ribs and muscle, skin). The rats were allowed to wake up after surgery and then extubated. They consumed a standard rat diet (Rodent Diet 5001, PMI Nutrition) and water ad libitum for the first 24 h; 24 h after surgery (MI), surviving rats were randomly assigned to the (n-6) or (n-3) diet. Rats were evaluated a minimum of twice daily and followed for 6 mo. Sudden death for this study was defined as the subsequent examination. In addition, at autopsy, no cause of death could be found upon gross examination.

Biochemical analysis of cardiac tissue. Hearts were obtained from 6-mo survivors consuming the (n-6) and (n-3) diets. Hearts were removed and wet weight obtained; the uninfarcted left ventricular tissue adjacent to the infarcted region was isolated, frozen in liquid nitrogen, and stored at −80°C until utilized for analysis. For biochemical analysis, the hearts were thawed on ice and homogenized using a polytron homogenizer in buffer containing 0.3 mol/L sucrose, 20 nmol/L L-Tris-HCl (pH 7.4), and 1 nmol/L dithiothreitol. Homogenates were centrifuged briefly at 500 × g to remove connective tissues and the supernatants were used for enzymatic assays.

Protein kinase A (PKA), protein kinase C (PKC), and calcium calmodulin–dependent kinase II (CaMKII) activities were measured using Upstate Biotechnology assay kits as previously described (29–32). Enzyme activity was expressed relative to protein content.

Expression of PKA, PKCa, and CaMKII was determined by Western immunoblotting using anti-PKA (Upstate Biotechnologies), anti-PKCa (Upstate Biotechnologies), and anti-CaMKII (Sigma Chemical) antibodies. Blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences), and bands were analyzed by densitometry using a KODAK 440CF Image documentation system (Eastman Kodak). Equal loading of protein was ensured using β-actin.

Levels of the ryanodine receptor calcium release channel-2 (RyR2) and its activation state (phosphorylation status) in equal quantities of homogenized left ventricular tissue were analyzed as previously described (33). The RyR2 receptors were immunoblotted with monoclonal anti-RyR2 antibodies to identify the protein and to determine the relative amounts of RyR2 in different samples. The blots were then stripped of antibodies (34). The stripped blots were blocked and reprobed with anti-phosphoserine-antibodies (Alexis) to determine the phosphorylation status of RyR2. Blots were developed using the ECL detection system (Amersham Biosciences) and bands were analyzed by densitometry using a KODAK 440CF Image documentation system (Eastman Kodak Company).

Statistical analysis. Lipid contents, kinase activities, and RyR2 levels are presented as means ± SD and were compared between the (n-6) and (n-3) diet groups using 2-sample, 2-sided Student’s tests for unequal variances. Survival was estimated using Kaplan-Meier procedures and was tested between diet groups using the Log-Rank test. Differences were considered significant at P < 0.05.

RESULTS

Tissue fatty acid composition. The (n-6) diet contained ∼58% (n-6) fatty acids in the form of linoleic acid (18:2), whereas it contained only 0.8% (n-3) fatty acids in the form of α-linolenic acid (18:3). In contrast, the (n-3) diet had only 3% (n-6) fatty acids, mainly linoleic and arachidonic acids (20:4), and 28% (n-3) fatty acids [mainly eicosapentaenoic acid (EPA); 20:5] and docosahexaenoic acid (DHA; 22:6)]. Compared with rats fed the (n-6) diet, rats fed the (n-3) diet had significantly lower concentrations of plasma and cardiac 18:2 and 20:4 (n-6) PUFAs (Table 2). On the other hand, the (n-3) diet group had significantly higher levels of plasma and cardiac 20:5 and 22:6 (n-3) PUFAs.

Sudden death. Rats fed the (n-3) diet survived longer than those fed the (n-6) diet (33/37 or 89.2% vs. 24/37 or 64.9% at 6 mo; P = 0.013; Fig. 1). The difference in survival represents a relative improvement of 37% at 6 mo. All rats were healthy before death and died from sudden death.

Cardiac weight and protein kinase activities. Relative heart weights did not differ between the groups [(n-3), 2.21 ± 0.03 mg/g body weight vs. (n-6), 2.29 ± 0.05 mg/g body weight]. Total kinase activity was 33% lower [(n-3) diet group compared with the (n-6) diet group (Fig. 2). PKA and CaMKII activities were ∼38% lower [(n-3) diet group compared with the (n-6) diet group (Fig. 2). PKA and CaMKII activities were ∼38% lower (P < 0.05) in

| TABLE 1 |
| Composition of experimental diets |
| (n-6) Diet | (n-3) Diet |
| Carbohydrate, % energy | 58.8 | 58.8 |
| Cornstarch, g/100 g | 25.0 | 25.0 |
| Maltodextrin, g/100 g | 5.0 | 5.0 |
| Sucrose, g/100 g | 30.0 | 30.0 |
| Cellulose, g/100 g | 5.0 | 5.0 |
| Protein, % energy | 19.6 | 19.6 |
| Casein, g/100 g | 20.2 | 20.2 |
| α-Methionine, g/100 g | 0.3 | 0.3 |
| Lipid, % energy | 21.7 | 21.7 |
| Corn oil, g/100 g | 10.0 | 1.0 |
| Fish oil, g/100 g | 0 | 9.0 |
| (n-6):(n-3) ratio | 72.5 | 0.11 |
| Mineral mix, g/100 g | 3.45 | 3.45 |
| Vitamin mix, g/100 g | 1.0 | 1.0 |
| dl-α-Tocopherol acetate, 50%, g/100 g | 0.03 | 0.03 |
| Tert-butylhydroquinone, g/100 g | 0.02 | 0.02 |

1 Differs between diets.
2 Follows the AIN-76A recommendations (55).
In contrast, the activity of PKC and the amounts of PKA, CaMKII, and PKCα protein (data not shown) did not differ between the groups. RyR2 activation. RyR2 activation (phospho-RyR2/total RyR2 ratios) was lower in the cardiac tissue of rats fed the (n-3) diet (0.21 ± 0.02) than in the (n-6) diet group (0.29 ± 0.01; P < 0.05). The RyR2 protein level did not differ between groups.

The macronutrient contents of the diets used in this study reflect those of a low-fat American diet (i.e., 20% protein, 22% lipid, 58% carbohydrate). The 2 diets were chosen because they reflect the extremes of (n-6) and (n-3) PUFA intake by humans, allowing for the greatest chance of detecting differences between the diets. The cardiac tissue membrane extracts from both diet groups had a 7- to 8-fold enrichment of DHA relative to EPA (Table 2). These results are similar to previous studies that indicated that DHA is the most abundant and primary storage form of (n-3) PUFAs in cell membranes (35–37). Wang et al. (37) showed that cardiac tissue expresses elongases that elongate PUFAs to DHA. In addition, these investigators reported that (n-3) PUFAs inhibit expression of fatty acid desaturases. The net effect is an accumulation of (n-3) PUFAs in the form of DHA. This observation is of particular interest because the fish oil–induced changes in PKA, CaMKII, and RyR2 phosphorylation are all membrane-associated processes. The results of this study suggest that DHA, not EPA, is primarily responsible for the decrease in sudden death in patients consuming (n-3) PUFAs in the diet or as supplements.

Some epidemiologic studies suggest that fish consumption reduces cardiovascular mortality (1–3), but others have not reported cardiovascular benefits from increased fish intake (9–11). Some investigators speculate that the conflicting results from epidemiologic studies reflect differences in definitions of cardiovascular deaths, confounding factors related to lifestyle,

### TABLE 2

| Fatty acid composition of plasma and heart from rats fed the (n-6) diet or (n-3) diet for 4 wk† |
|----------------------------------------|---------|---------|---------|
| Plasma                                | Heart   |
| (n-6)                                 | (n-3)   | (n-6)   | (n-3)   |
| 18:2(n-6)                             | 38.9 ± 4.5* | 7.9 ± 0.8 | 25.3 ± 1.2* | 12.3 ± 1.3 |
| 20:4(n-6)                             | 7.31 ± 0.4*  | 5.5 ± 0.5  | 23.0 ± 1.3*  | 11.8 ± 0.5  |
| 20:5(n-3)                             | 5.11 ± 0.5*  | 11.9 ± 1.3 | <0.5*       | 3.2 ± 0.3   |
| 22:6(n-3)                             | 0.8 ± 0.04*  | 9.6 ± 1.1   | 4.2 ± 0.5   | 24.0 ± 0.5  |
| Saturated                             | 24.0 ± 2.0*  | 37.0 ± 3.0  | 33.1 ± 0.8*  | 37.1 ± 1.7  |
| Monounsaturated                       | 17.0 ± 2.0*  | 9.2 ± 0.7   | 8.0 ± 1.0*   | 6.4 ± 0.5   |
| Σ (n-6)                               | 46.3 ± 5.0*  | 13.4 ± 1.3  | 48.3 ± 1.9*  | 24.1 ± 1.3  |
| Σ (n-3)                               | 5.9 ± 0.6*   | 21.5 ± 2.4  | 4.2 ± 0.5*   | 27.3 ± 0.7  |
| (n-6):(n-3)                           | 7.9 ± 1.8*   | 0.6 ± 0.1   | 11.8 ± 1.6*  | 0.9 ± 0.1   |

† Values are means ± SD, n = 5. *Different from (n-3), P < 0.05.
inadequate experimental design, poor estimation of fish intake, inadequate estimation of the type of fish consumed, small fractions of some populations reporting little or no fish intake, and variability of the study populations.

Subsequently, randomized, controlled clinical trials were performed to evaluate more fully the potential cardiovascular benefits from dietary (n-3) PUFAs. The results from these trials were also discordant. Mortality was reduced using diet in the Diet and Reinfarction Trial (DART) (5,38) and the trial of De Longeril et al. (7). Fish oil supplements decreased mortality in the Indian Experiment of Infarct Survival trial (6), the GISST-Prevenzione trial (4,39), and the trial of Leaf et al. (8) in patients with cardioverter/defibrillators. The primary decrease in mortality resulted from a decrease in sudden deaths (39). In support of the decrease in sudden death, (n-3) PUFAs were shown to decrease the incidence of arrhythmias in experimental models (40–47). In contrast, a number of prospective randomized controlled trials found no cardiovascular benefits from increased dietary intake of (n-3) PUFAs using fish oils after MI (12) or implantation of cardioverter/defibrillators (15) or by α-linolenic acid (13,14). Thus, further studies are warranted to confirm the beneficial cardiovascular effects of (n-3) PUFAs.

Most causes of sudden cardiac death are related to abnormal cardiac electrical activity resulting in ventricular arrhythmias, bradyarrhythmias, or asystole (21,48,49). Cardiac electrical activity is dependent upon numerous cellular processes that include membrane structure, ion channel activity, signaling pathways, and enzyme activities. We chose to study the 3 kinases and RyR2 activation because each protein has been linked to the generation of cardiac arrhythmias (19–21,50–52).

Previous animal studies by McLennan and co-workers (40,41) and Leaf and co-workers (42,43) established the short-term antiarrhythmic effects of (n-3) fatty acids. Subsequent studies demonstrated that (n-3) fatty acids modulate the conductance currents, especially the voltage-dependent sodium and L-type calcium channels in heart cell membranes (44–47). Our data extend these observations and suggest that modulation of phosphorylation may be one mechanism responsible for alterations in ionic conductance and arrhythmia generation after increased (n-3) fatty acid intake.

In this study, we did not measure levels of blood lipoproteins (i.e., LDL, HDL, VLDL) or cholesterol due to limited quantities of blood. We doubt that alterations in lipoproteins were responsible for our results due to the short-term of the study. We also did not directly access cardiac function in the rats. Echocardiographic assessment of contraction is difficult in rats and we were not able to obtain reliable measurements of contractility. Due to technological limitations, we were also not able to monitor the rats for arrhythmias (i.e., using telemetry). Thus, we based sudden death on clinical criteria (similar to criteria used in clinical studies). Because all rats were healthy within 12 h of their death and autopsies did not reveal a specific cause of death, we believe that cardiac arrhythmias were the most likely cause of death. At the time of tissue harvesting, we checked each heart for the site of ligation and the presence of an infarction. The size of the infarcts (assessed visually) and cardiac weight:body weight ratios were similar in each group. We also did not use sham-operated rats as controls because we were interested in rats after MI. Thus, we do not know whether the rats developed myocardial hypertrophy. However, our values for cardiac weight:body weight ratios are similar to those in the published literature (53,54); the 8-mo mortality in rats fed normal diets is ~0. Thus, mortality in our rats undergoing coronary ligation was higher than that of normal rats.

In summary, this is the first long-term dietary outcome study of sudden death using a rat model of MI. We report that (n-3) PUFAs in the diet significantly reduce the occurrence of sudden death in the months after MI in rats. Other novel findings from the study are the decreases in protein kinase activities and RyR2 activation induced by feeding (n-3) PUFAs. Our study suggests that these proteins may represent novel targets for the treatment of cardiac arrhythmias. Clearly, further studies are warranted to investigate the cellular mechanisms for the cardioprotective actions of (n-3) PUFAs.

LITERATURE CITED