Dietary Omega-3 Fatty Acids Promote Arrhythmogenic Remodeling of Cellular Ca\textsuperscript{2+} Handling in a Postinfarction Model of Sudden Cardiac Death

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Abstract

It has been proposed that dietary omega-3 polyunsaturated fatty acids (n-3 PUFAs) can reduce the risk of ventricular arrhythmias in post-MI patients. Abnormal Ca\textsuperscript{2+} handling has been implicated in the genesis of post-MI ventricular arrhythmias. Therefore, we tested the hypothesis that dietary n-3 PUFAs alter the vulnerability of ventricular myocytes to cellular arrhythmia by stabilizing intracellular Ca\textsuperscript{2+} cycling. To test this hypothesis, we used a canine model of post-MI ventricular fibrillation (VF) and assigned the animals to either placebo (1 g/day corn oil) or n-3 PUFAs (1-4 g/day) groups. Using Ca\textsuperscript{2+} imaging techniques, we examined the intracellular Ca\textsuperscript{2+} handling in myocytes isolated from post-MI hearts resistant (VF-) and susceptible (VF+) to VF. Frequency of occurrence of diastolic Ca\textsuperscript{2+} waves (DCWs) in VF+ myocytes from placebo group was significantly higher than in placebo-treated VF- myocytes. In contrast, VF- myocytes from the n-3 PUFAs group had a significantly higher frequency of DCWs than myocytes from the placebo group. In addition, n-3 PUFAs treatment increased beat-to-beat alterations in the amplitude of Ca\textsuperscript{2+} transients (Ca\textsuperscript{2+} alternans) in VF-mycocytes. These n-3 PUFAs effects in VF- myocytes were associated with an increased Ca\textsuperscript{2+} spark frequency and reduced sarcoplasmic reticulum Ca\textsuperscript{2+} content, indicative of increased activity of ryanodine receptors. Thus, dietary n-3 PUFAs do not alleviate intracellular Ca\textsuperscript{2+} cycling remodeling in myocytes isolated from post-MI VF+ hearts. Furthermore, dietary n-3 PUFAs increase vulnerability of ventricular myocytes to cellular arrhythmia in post-MI VF-hearts by destabilizing intracellular Ca\textsuperscript{2+} handling.

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Introduction

Cardiac arrhythmias are recognized as a major factor contributing to morbidity and mortality in patients with healed myocardial infarction (MI). The search for an effective anti-arrhythmic therapy remains a major unmet challenge. Initial observational and interventional studies indicated that dietary omega-3 polyunsaturated fatty acids (n-3 PUFAs) may be effective in preventing cardiac arrhythmias [1–3]. However, more recent clinical and animal studies reported mixed results as to the anti-arrhythmic effects of n-3 PUFAs [4–7]. To explain the apparent heterogeneity of the results, it has been suggested that the effectiveness of n-3 PUFAs treatment might depend on the mechanism of cardiac arrhythmia (triggered vs. reentry), and on the route of n-3 PUFAs administration (infused, free circulating vs. dietary, lipid incorporated)[6,8]. Abnormal regulation of intra-mycyte Ca\textsuperscript{2+} handling observed in various cardiac disease settings, including post-MI hearts, has been implicated in the genesis of both triggered and reentrant arrhythmias [9–13]. Mechanistically, dysregulation of Ca\textsuperscript{2+} cycling that is manifested by increased frequency of diastolic Ca\textsuperscript{2+} waves (DCWs) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger-mediated delayed after-depolarizations (DADs) is usually associated with triggered arrhythmia mechanisms. Additionally, remodeling of Ca\textsuperscript{2+} handling that results in increased susceptibility to beat-to-beat alterations in the amplitude of Ca\textsuperscript{2+}.

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transients (Ca²⁺ alternans), and thereby an increased dispersion of repolarization, can be linked to reentrant mechanisms of arrhythmia. Therefore, the overall success of anti-arrhythmic treatment with n-3 PUFAs may depend upon its effects on intra-mycocyte Ca²⁺ handling.

In cellular studies, the acute application of free n-3 PUFAs consistently depressed intracellular Ca²⁺ handling, by reducing the frequency Ca²⁺ sparks [14], Ca²⁺ after-transients [15] and Ca²⁺ influx via the L-type Ca²⁺ channels [16–19], as well as decreasing levels of systolic and diastolic Ca²⁺ [15,17,19], and inhibiting the activity of reconstituted ryanodine receptors (RyR2s) [14,20], a sarcoplasmic reticulum Ca²⁺ release channel. These data indicate that free n-3 PUFAs can be effective in suppressing diastolic Ca²⁺ waves and in preventing triggered arrhythmia [15,19]. Similarly, dietary n-3 PUFAs were shown to suppress arrhythmic contractile activity and Ca²⁺ after-transients in myocytes isolated from control hearts [21,22]. However the effects of chronic dietary n-3 PUFAs on intracellular Ca²⁺ handling in diseased myocytes remain to be determined.

In the present study, we used a well-characterized canine model of healed MI [23] to investigate the effects of dietary n-3 PUFAs (1–4 g/day docosahexaenoic acid + eicosapentaenoic acid ethyl esters) on intracellular Ca²⁺ cycling in isolated ventricular myocytes. Using a standardized exercise plus ischemia test, post-MI animals were stratified for susceptibility to ventricular fibrillation (VF) into susceptible (VF⁺) and resistant (VF⁻) groups. We show that dietary n-3 PUFAs produced alterations in intracellular Ca²⁺ cycling in post-MI myocytes that are consistent with a pro- rather than an anti-arrhythmic effect.

Materials and Methods

The principles governing the care and use of animals as expressed by the Declaration of Helsinki, and as adopted by the American Physiological Society, were followed at all times during this study. In addition, the Ohio State University Institutional Animal Care and Use Committee approved all the procedures used in this study.

Model

A description of the model, n-3 PUFA treatment protocol, and previous in vivo results have been described in detail [7]. Briefly, heartworm free mixed breed dogs (2-3 y old) were anesthetized and instrumented to measure a ventricular electrogram and coronary blood flow as previously described [23–25]. A hydraulic vascular occluder was placed around the left circumflex coronary artery and used to induce acute myocardial ischemia during the exercise plus ischemia test as described below. The left anterior descending coronary artery was also isolated during the instrumentation surgery and a two-stage occlusion of this artery was then performed approximately one-third the distance from its origin in order to produce an anterior wall myocardial infarction (~16% of left ventricular mass [23]). Three-to-four weeks after the production of the myocardial infarction, the susceptibility to ventricular fibrillation (VF) was tested as previously described [23–25]. The animals ran on a motor-driven treadmill while workload progressively increased until a heart rate of 70% of maximum (approximately 210 beats/min) had been achieved. During the last minute (on average during the 18th minute) of exercise, the left circumflex coronary artery was occluded, the treadmill stopped and the occlusion maintained for an additional minute (total occlusion time = 2 min.). The exercise plus ischemia test reliably induced ventricular flutter that rapidly deteriorated into VF. Therefore, large defibrillation electrodes were placed across the animal’s chest so that electrical defibrillation could be achieved with a minimal delay but only after the animal was unconscious (10-20 s after the onset of VF). The occlusion was immediately released if VF occurred.

Omega-3 protocol

The dogs were placed on a diet that did not contain any n-3 PUFAs beginning one week prior to the instrumentation surgery and were maintained on this diet until the end of the study (~ 4 months). After the pre-treatment data collection (3 - 4 weeks after the surgery), the dogs were then randomly assigned to the following groups: placebo (n = 17: VF⁺, n = 9; VF⁻, n = 8); n-3 PUFA (1–4 g/day, n = 45: VF⁺ n = 22; VF⁻, n = 23). The dogs were given supplements similar to those used in the GISSI-Prevenzione study [26]. The n-3 PUFA group received 465 mg ethyl eicosapentaenoate, EPA + ethyl docosahexaenoate, DHA, 375 mg per 1 g capsule (Lovaza®, GlaxoSmithKline, Research Triangle Park, NC); doses of 1, 2, 4 grams were given. As no dose-dependent differences were found, data for all doses were grouped together. The placebo was corn oil (1 g, 58% linoleic acid + 28% oleic acid). The capsules were given per os prior to the daily feeding (between 8:00 and 10:00 AM each day, 7 days per week for 3 months). As previously reported [7,27], dietary EPA + DHA ethyl esters elicited significant increases in left ventricle n-3 PUFA content, reaching a peak between 8 and 12 weeks.

Cellular Ca²⁺ imaging

Myocytes were isolated distant from the infarction zone of the left ventricular midmyocardium as described previously [28]. For present study cells were isolated from normal control dogs (n=8, no surgery, no MI, untreated), n-3 PUFAs treated sham controls (n=3, no MI), untreated VF⁻ (n=2), placebo treated VF⁻ (n=3) and VF⁺ (n=3) dogs, and n-3 PUFA treated VF⁻ (n=3) and VF⁺ (n=4) dogs. Electrical field stimulation experiments were performed using the following external solution (in mM): 140 NaCl, 5.4 KCl, 2.0 CaCl₂, 0.5 MgCl₂, 10 HEPES, and 5.6 glucose (pH 7.4). Intracellular Ca²⁺ imaging was performed using an Olympus Fluoview 1000 confocal microscope. Rhod-2 Ca²⁺ indicator was used to monitor cytosolic Ca²⁺ in intact myocytes. Cells were incubated with 10 μM Rhod-2 AM (Life Technologies, Grand Island, NY) for 25 min at room temperature. Amplitude of Ca²⁺ alternans was defined as 100*(A₁/A₂)*100 %, where A₁ and A₂ are amplitudes of two consecutive Ca²⁺ transients. Ca²⁺ sparks were studied in saponin-permeabilized myocytes using 30 μM Fluo-3 (Life Technologies, Grand Island, NY) and the following intracellular solution: (mM) 120 potassium aspartate, 20 KCl, 3 MgATP, 10 phosphocreatine, 5 U ml⁻¹ creatine phosphokinase,
0.5 EGTA (pCa 7) and 20 HEPES (pH 7.2). Ca\textsuperscript{2+} sparks were detected and analyzed using a computer algorithm described previously [29]. Image processing and analysis was performed using ImageJ (National Institutes of Health; http://rsbweb.nih.gov/ij/) and Origin 7.0 (OriginLab Corporation, Northampton, MA) programs.

**Western Blotting**

The levels of proteins involved in Ca\textsuperscript{2+} cycling and their phosphorylation were assessed by immunoblot analysis using 20-40 mg of homogenates from left ventricular tissue samples as described previously [30]. Primary antibodies used were: anti-phospholamban (PLB), anti- Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX1), and anti-phospho-PLB-S16 from Millipore (Billerica, MA); anti-SERCA2a from Sigma-Aldrich (St.Louis, MO); anti-RyR2 and anti-Cav1.2 from ThermoScientific (Waltham, MA); anti-phospho-PLB-T17 from Santa Cruz (Dallas, TX). Anti-phospho-RyR2-S2030 antibody was raised against (CG) TIRGRLLS(PO4)LVEKVTYLKKCONH\textsubscript{2} (YenZym Abs, South San Francisco, CA). Custom-made anti-phospho-RyR2-S2808 and anti-phospho-RyR2-S2814 were from Phosphosolutions (Aurora, CO)[30]. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Abcam (Cambridge, MA). Expression levels of RyR2, SERCA2A, PLB, Cav1.2 channels, and NCX1 were assessed after normalization to the loading control, GAPDH. Phosphorylation levels of RyR2 and PLB were analyzed following normalization to RyR2 or PLB protein levels assessed from gels run in parallel. Blots were developed with Super Signal West Pico (Pierce) and quantified using ImageJ (National Institutes of Health) and Origin 7 (OriginLab, Northampton, MA) software.

**Statistical Analysis**

Results are presented as mean±S.E.M. Statistical significance was evaluated using either Student's t test or one way ANOVA with Tukey's post hoc test. The proportion of cells displaying DCWs or Ca\textsuperscript{2+} alternans was compared using Fisher's exact test. A P value of <0.05 was considered significant.

**Results**

**Dietary n-3 PUFA\textquotesingle s do not stabilize intracellular Ca\textsuperscript{2+} cycling in VF+ myocytes and increase susceptibility of VF- myocytes to pro-arrhythmic diastolic Ca\textsuperscript{2+} waves**

Recordings of cytosolic Ca\textsuperscript{2+} in field-stimulated myocytes in the presence of β-adrenergic receptor agonist isoproterenol (100 nM) were used to analyze susceptibility of ventricular myocytes to DCWs. On average, the frequency of occurrence of DCWs was not different in untreated controls and VF-myocytes from placebo group (Figure 1 A, B, D). DCWs were more frequent (P<0.05) in field-stimulated VF+ myocytes from the placebo-treated group than in VF- myocytes from corresponding group (Figure 1 B-E). n-3 PUFA treatment did not affect the rate of the occurrence of DCWs either in control (P=0.5) or in VF+ (P=0.2) myocytes (Figure 1 A, C, D, E). Conversely, in VF- myocytes n-3 PUFA treatment significantly increased (P<0.05 vs. placebo) frequency of DCWs (Figure 1 B, D). Furthermore, the proportion of myocytes displaying DCWs increased more than three-fold (P<0.01) in VF-myocytes treated with n-3 PUFA\textquotesingle s when compared to placebo-treated cells (Figure 1 E).

**Dietary n-3 PUFA\textquotesingle s increase susceptibility of VF- myocytes to pro-arrhythmic Ca\textsuperscript{2+} alternans**

To investigate whether the effects of dietary n-3 PUFA\textquotesingle s on VF- myocytes were associated with Ca\textsuperscript{2+}-dependent arrhythmogenic substrate, we studied the amplitude and rate-dependence of Ca\textsuperscript{2+} alternans in VF- myocytes from placebo and n-3 PUFA group [9,31]. As demonstrated in Figure 2, both untreated controls and placebo-treated VF- myocytes did not normally exhibit Ca\textsuperscript{2+} alternans at 0.5 and 1 Hz frequency of field stimulation. In contrast, following n-3 PUFA treatment 75 % of VF- myocytes displayed Ca\textsuperscript{2+} alternans at 1 Hz (Figure 2 B). This increase in a number of cells displaying alternans was also associated with a significant increase (P<0.05 vs. placebo) in average amplitude of Ca\textsuperscript{2+} alternans recorded in VF- from n-3 PUFA\textquotesingle s treated group at 1 Hz (Figure 2 B, C, D). These data suggest that dietary n-3 PUFA\textquotesingle s may enhance the dynamic substrate for arrhythmia in VF- hearts.

**Effect of dietary n-3 PUFA\textquotesingle s on intracellular Ca\textsuperscript{2+} handling in VF- myocytes is associated with the increased ryanodine receptor (RyR2) activity**

We further characterized the effect of dietary n-3 PUFA\textquotesingle s on properties of intracellular Ca\textsuperscript{2+} handling in VF- myocytes by measuring the frequency of Ca\textsuperscript{2+} sparks. As shown in Figure 3 and Table 1 Ca\textsuperscript{2+} sparks frequency was significantly higher in untreated VF- myocytes when compared to control. However, even greater increases in Ca\textsuperscript{2+} spark frequency were observed in VF- myocytes from the n-3 PUFA treated group (Figure 3 A, C; table 1). To assess possible mechanisms underlying the n-3 PUFA-induced augmented Ca\textsuperscript{2+} spark activity in VF- myocytes, we studied SR Ca\textsuperscript{2+} content ([Ca\textsuperscript{2+}]\textsubscript{SR}) by measuring the amplitude of Ca\textsuperscript{2+} transients evoked by 10 mM caffeine. As shown in Figure 3 (B and D), [Ca\textsuperscript{2+}]\textsubscript{SR} was significantly lower in n-3 PUFA-treated VF- myocytes compared to untreated control and VF- myocytes, respectively. More frequent Ca\textsuperscript{2+} sparks at lower [Ca\textsuperscript{2+}]\textsubscript{SR} indicate increased RyR2 functional activity in VF-myocytes from n-3 PUFA-treated group.

Next, we assessed whether changes in expression and phosphorylation levels of proteins involved in intracellular Ca\textsuperscript{2+} cycling occur following chronic dietary supplementation with n-3 PUFA\textquotesingle s. Dietary n-3 PUFA\textquotesingle s did not significantly affect expression of RyR2, SR Ca\textsuperscript{2+} ATPase, phospholamban (PLB), alpha subunit of cardiac L-type Ca\textsuperscript{2+} channels, and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger either in control or in VF- ventricular preparations (Figure 4 A-C, table 2). We also observed no significant alterations in RyR2 phosphorylation at well-established phosphorylation sites (Ser-2808, Ser-2814, and Ser-2030) [32,33] in n-3 PUFA-treated groups (Figure 4 B, table 2). Finally, phosphorylation levels of PLB at Ser-16 and Thr-17 were also unaffected by n-3 PUFA treatment (Figure 4 C, table 2).
Figure 1. Dietary n-3 PUFAs induce pro-arrhythmic remodeling of intracellular Ca\(^{2+}\) handling in VF-myocytes. Representative line-scan images and corresponding profiles of Rhod-2 fluorescence during periodic (0.3 Hz) electrical stimulation recorded in myocytes from placebo/untreated and n-3 PUFA-treated controls (A), VF- (B) and VF+ (C) groups, respectively. Data were obtained in the presence of 100 nM isoproterenol, a β-adrenergic receptor agonist. D, Average frequency of DCWs (per second) was: 0.11±0.03 (n=15) and 0.14±0.03 (n=40) in control untreated and n-3 PUFA-treated myocytes, respectively (P=0.5); 0.07±0.03 (n=20) and 0.21±0.04 (n=20) in VF- myocytes from placebo and n-3 PUFAs groups, respectively (P=0.014); 0.23±0.05 (n=8) and 0.32±0.05 (n=8), in VF+ myocytes from placebo and n-3 PUFAs groups, respectively (P=0.22). *, P<0.05 vs. VF- placebo; †, P<0.05 vs. n-3 PUFA-treated controls. E, Bar graph shows proportion of myocytes displaying DCWs. In control groups DCWs were recorded in 9 out of 15 untreated cells and in 24 out of 40 n-3 PUFA-treated cells, respectively. In VF- groups DCWs were recorded in 4 out of 20 placebo-treated cells and in 8 out of 20 n-3 PUFA-treated cells, respectively. In VF+ groups DCWs were recorded in 7 out of 8 placebo-treated cells and in 8 out of 8 n-3 PUFA-treated cells, respectively.

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Discussion

In the present study we tested the hypothesis that dietary n-3 PUFAs would stabilize intracellular Ca$^{2+}$ cycling in ventricular myocytes isolated from post-MI hearts. The major findings are as follows: a) dietary n-3 PUFAs were not effective in inhibiting DCWs in ventricular myocytes isolated from VF+ animals; b) dietary n-3 PUFAs caused marked destabilization of intracellular Ca$^{2+}$ cycling in myocytes from VF- animals manifested as an increased rate of occurrence of DCWs and increased amplitude of Ca$^{2+}$ alternans; c) effects of dietary n-3 PUFAs observed in VF- myocytes were associated with
Figure 3. Dietary n-3 PUFAs increase frequency of Ca\(^{2+}\) sparks in VF- myocytes. A, Representative line-scan images of Ca\(^{2+}\) sparks recorded in saponin-permeabilized myocytes from indicated groups. Insets show scaled up image of Ca\(^{2+}\) spark with corresponding time-dependent fluorescence profile. B, Representative traces of Ca\(^{2+}\) transients evoked by 10 mM caffeine recorded in permeabilized myocytes from indicated groups. C, Average Ca\(^{2+}\) spark frequency (in 100 µm\(^{-1}\) s\(^{-1}\)) was 1.23±0.13 (n=52) in untreated control myocytes, 2.01±0.19 (n=47) and 3.31±0.38 (n=45) in untreated and n-3 PUFA-treated VF- myocytes, respectively. D, Average amplitude of caffeine-induced Ca\(^{2+}\) transients ([Ca\(^{2+}\)]\(_{\text{CAFF}}\), ΔF/F\(_{0}\)) was 2.76±0.34 (n=7) in untreated control myocytes, 2.19±0.11 (n=5) and 1.42±0.06 (n=4) in untreated and n-3 PUFA-treated VF- myocytes, respectively. *, P<0.05 vs. control; †, P<0.05 vs. VF- untreated.

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enhanced RyR2 activity. These cellular findings may explain our previous in vivo observation that dietary n-3 PUFA not only failed to reduce the risk for ventricular tachyarrhythmias in VF+ dogs but actually increased arrhythmia formation in VF- dogs [7].

Intracellular Ca²⁺ dysregulation is recognized as an important factor contributing to the genesis of various forms of cardiac arrhythmias. Remodeling of intracellular Ca²⁺ cycling leading to increased occurrences of spontaneous Ca²⁺ releases and diastolic Ca²⁺ waves is typically associated with triggered arrhythmias [10,11,13]. Alterations in intracellular Ca²⁺ handling resulting in beat-to-beat variations in the amplitude of Ca²⁺ transient (Ca²⁺ alternans) are believed to contribute to reentrant excitation, providing an additional form of proarrhythmic dysregulation [9–11]. Using canine post-MI model of sudden cardiac death we previously showed that ventricular myocytes isolated from VF+ hearts had higher susceptibility to both DCWs than myocytes isolated from normal hearts. In the present study most likely results from indirect effects. We acknowledge that present study has some limitations that results in increases in both free circulating and lipid incorporated PUFAs [6,8]. Indeed, most cellular data supporting an anti-arrhythmic effect of PUFAs were obtained from studies that evaluated the effects of the acute application of free n-3 PUFAs. Thus, acute application of free n-3 PUFAs invariably resulted in inhibitory effects on membrane excitability and Ca²⁺ handling [14–19] [reviewed in 6,35]. Consistent with these in vitro studies, acute infusion of free n-3 PUFAs reduced in vivo susceptibility to VF in our canine post-MI model [36].

### Table 1. Properties of Ca²⁺ sparks in saponin-permeabilized ventricular myocytes.

|                | Control untreated | VF- untreated | VF- n-3 PUFA untreated |
|----------------|-------------------|---------------|------------------------|
|                | n=62              | n=47          | n=45                   |
| Amplitude (ΔF/F₀) | 0.74±0.02         | 0.64±0.01†     | 0.70±0.01†             |
| Frequency (sparks/100 µs) | 1.23±0.13         | 2.01±0.19†     | 3.31±0.38††          |
| FWHM (ms)      | 21.9±0.3          | 23.8±0.8      | 18.0±0.4‡‡            |
| FWHM (µm)      | 2.07±0.05         | 1.96±0.04     | 2.06±0.04             |
| Time to peak (ms) | 10.50±0.39        | 9.71±0.43     | 8.32±0.27†‡          |
| FDHM, full duration at half maximum; FWHM, full width at half maximum. N, number of cells studied. * P<0.05 vs. control untreated; † P<0.05 vs. VF- untreated.

Animal studies addressing the effects of dietary n-3 PUFAs have produced more heterogeneous results [reviewed in 6,35]. For example, dietary n-3 PUFAs inhibited ischemia and reperfusion arrhythmias in rat hearts [37] but promoted arrhythmias during acute myocardial ischemia in pig hearts [38] and increased in vivo susceptibility to VF in dogs with healed MI [7]; the very same animals from which myocytes were obtained for the present studies. In ventricular myocytes isolated from control animals, incorporated n-3 PUFAs did not significantly affect Ca²⁺ transients under baseline conditions, but reduced both arrhythmogenic Ca²⁺ after-transients and arrhythmogenic contractile activity evoked by beta-adrenergic receptor stimulation [21,22]. We previously showed that incorporated n-3 PUFAs did not change Ca²⁺ transients under baseline conditions in myocytes isolated from post-MI canine hearts [27]. To the best of our knowledge, the present study is the first to address the effect of dietary n-3 PUFAs on arrhythmogenic properties of intracellular Ca²⁺ cycling in the setting of healed MI with known in vivo susceptibility to cardiac arrhythmias. In our experiments, dietary n-3 PUFAs resulted in severe pro-arrhythmic alterations in intracellular Ca²⁺ cycling in VF- myocytes (Figures 1-3), whereas susceptibility of VF+ myocytes to DCWs, already high in the placebo group, was not significantly affected by n-3 PUFAs (Figure 1 C-E). It is worthwhile to note that dietary n-3 PUFAs did not affect the stability of intracellular Ca²⁺ cycling in ventricular myocytes isolated from controls (Figure 1 A, D, E) suggesting that the pro-arrhythmic effect may depend on cellular substrate (magnitude and mechanisms of cellular remodeling due to MI).

The n-3 PUFA influence on ion channel activity has been attributed to the direct interactions with the channel proteins and indirect effects on membrane fluidity and intracellular signaling [5,6,8]. Given that the acute application of n-3 PUFA inhibits RyR2s [14,20], enhanced activity of RyR2s observed in the present study most likely results from indirect effects. We did not find evidence that dietary n-3 PUFA alter expression levels of proteins involved in cardiac Ca²⁺ signaling [5,6,8]. The n-3 PUFA influence on ion channel activity has been attributed to the direct interactions with the channel proteins and indirect effects on membrane fluidity and intracellular signaling [5,6,8]. Given that the acute application of n-3 PUFA inhibits RyR2s [14,20], enhanced activity of RyR2s observed in the present study most likely results from indirect effects. We did not find evidence that dietary n-3 PUFA alter expression levels of proteins involved in cardiac Ca²⁺ signaling [5,6,8]. Given that the acute application of n-3 PUFA inhibits RyR2s [14,20], enhanced activity of RyR2s observed in the present study most likely results from indirect effects. We did not find evidence that dietary n-3 PUFA alter expression levels of proteins involved in cardiac Ca²⁺ signaling [5,6,8]. Given that the acute application of n-3 PUFA inhibits RyR2s [14,20], enhanced activity of RyR2s observed in the present study most likely results from indirect effects. We did not find evidence that dietary n-3 PUFA alter expression levels of proteins involved in cardiac Ca²⁺ signaling [5,6,8]. Given that the acute application of n-3 PUFA inhibits RyR2s [14,20], enhanced activity of RyR2s observed in the present study most likely results from indirect effects. We did not find evidence that dietary n-3 PUFA alter expression levels of proteins involved in cardiac Ca²⁺ signaling [5,6,8]. Given that the acute application of n-3 PUFA inhibits RyR2s [14,20], enhanced activity of RyR2s observed in the present study most likely results from indirect effects. We did not find evidence that dietary n-3 PUFA alter expression levels of proteins involved in cardiac Ca²⁺ signaling [5,6,8].

### Study limitation

We acknowledge that present study has some limitations that could affect the interpretation of the results. Due to technical reasons all cellular experiments were performed at...
Figure 4. Dietary n-3 PUFAs do not affect expression and phosphorylation levels of proteins involved in cardiac Ca\textsuperscript{2+} cycling. A-C, Representative immunoblots of left ventricle homogenates prepared from placebo and n-3 PUFA-treated control (sham) and VF- groups. NCX1, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger type 1; SERCA2a, cardiac isoform of SR Ca\textsuperscript{2+}-ATPase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; PLN, phospholamban; Cav1.2, α1C subunit of L-type Ca\textsuperscript{2+} channel. Data were obtained using 2-4 heart samples. Quantitative analysis is presented in Table 2.

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room temperature (22-24°C) and ambient O2 tension (~20%) in contrast to physiological temperature (37°C) and O2 tension (~5%). Therefore, our experimental conditions could influence dynamics of intracellular Ca2+ cycling, fluidity of the membrane and potentially could alter the intracellular effects of incorporated n-3 PUFAs. Finally, efficacy of incorporated n-3 PUFAs may be different in canine and human hearts.

Conclusions

In the present study, we have demonstrated that increases in left ventricle n-3 PUFA content mediated by dietary intake of EPA +DHA ethyl esters similar to those noted in patients [26,40] were associated with a significant increases in frequency of Ca2+ sparks in myocytes from post-MI (VF-) hearts. The increased frequency of Ca2+ sparks along with the reduced SR Ca2+ content observed in VF- myocytes suggest that incorporated n-3 PUFAs increased sensitivity of ryanodine receptors to SR Ca2+ in diseased hearts. We further demonstrated that dietary n-3 PUFA supplements were associated with a high predisposition of both VF- and VF+ myocytes to DCWs in response to β-adrenergic receptor stimulation. Thus, we conclude that incorporated n-3 PUFAs produce disturbances in Ca2+ cycling that would increase rather than decrease the risk for ventricular tachyarrhythmias in post-MI hearts.

Author Contributions

Conceived and designed the experiments: AEB DT CAC SG GEB. Performed the experiments: AEB HH IMB DT GEB RT. Analyzed the data: AEB HH DT GEB. Contributed reagents/materials/analysis tools: CAC GEB. Wrote the manuscript: AEB DT CAC SG GEB.

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