Dietary Omega-3 Fatty Acids Promote Arrhythmogenic Remodeling of Cellular Ca²⁺ Handling in a Postinfarction Model of Sudden Cardiac Death

Andriy E. Belevych[1,2*], Hsiang-Ting Ho[1,2], Radmila Terentyeva[1,2,4], Ingrid M. Bonilla[3], Dmitry Terentyev[1,2,4], Cynthia A. Carnes[1,2,3], Sandor Gyorke[1,2], George E. Billman[1,2]

1 Dorothy M. Davis Heart and Lung Research Institute, the Ohio State University, Columbus, Ohio, United States of America, 2 Department of Physiology and Cell Biology, College of Medicine, the Ohio State University, Columbus, Ohio, United States of America, 3 College of Pharmacy, the Ohio State University, Columbus, Ohio, United States of America, 4 Department of Medicine, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, Rhode Island, United States of America

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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ imaging techniques, we examined the intracellular Ca²⁺ handling in myocytes isolated from post-MI hearts resistant (VF-) and susceptible (VF+) to VF. Frequency of occurrence of diastolic Ca²⁺ waves (DCWs) in VF+ myocytes from placebo group was significantly higher than in placebo-treated VF- myocytes. n-3 PUFA treatment did not decrease frequency of DCWs in VF+ myocytes. In contrast, VF- myocytes from the n-3 PUFA group had a significantly higher frequency of DCWs than myocytes from the placebo group. In addition, n-3 PUFA treatment increased beat-to-beat alterations in the amplitude of Ca²⁺ transients (Ca²⁺ alternans) in VF- myocytes. These n-3 PUFAs effects in VF- myocytes were associated with an increased Ca²⁺ spark frequency and reduced sarcoplasmic reticulum Ca²⁺ content, indicative of increased activity of ryanodine receptors. Thus, dietary n-3 PUFAs do not alleviate intracellular Ca²⁺ 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 Ca²⁺ handling.

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-myocyte Ca²⁺ 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 Ca²⁺cycling that is manifested by increased frequency of diastolic Ca²⁺ waves (DCWs) and Na⁺/Ca²⁺ exchanger-mediated delayed after-depolarizations (DADs) is usually associated with triggered arrhythmia mechanisms. Additionally, remodeling of Ca²⁺ handling that results in increased susceptibility to beat-to-beat alterations in the amplitude of Ca²⁺

transients (Ca^{2+} 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-myocyte Ca^{2+} handling.

In cellular studies, the acute application of free n-3 PUFAs consistently depressed intracellular Ca^{2+} handling, by reducing the frequency Ca^{2+} sparks [14], Ca^{2+} after-transients [15] and Ca^{2+} influx via the L-type Ca^{2+} channels [16–19], as well as decreasing levels of systolic and diastolic Ca^{2+} [15,17,19], and inhibiting the activity of reconstituted ryanodine receptors (RyR2s) [14,20], a sarcoplasmic reticulum Ca^{2+} release channel. These data indicate that free n-3 PUFAs can be effective in suppressing diastolic Ca^{2+} waves and in preventing triggered arrhythmia [15,19]. Similarly, dietary n-3 PUFAs were shown to suppress arrhythmic contractile activity and Ca^{2+} after-transients in myocytes isolated from control hearts [21,22]. However the effects of chronic dietary n-3 PUFAs on intracellular Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 PUFAs 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 CaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, and 5.6 glucose (pH 7.4). Intracellular Ca^{2+} imaging was performed using an Olympus Fluoview 1000 confocal microscope. Rhod-2 Ca^{2+} indicator was used to monitor cytosolic Ca^{2+} 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 Ca^{2+} alternans was defined as $100-(A_2/A_1)*100$ (%), where A_1 and A_2 are amplitudes of two consecutive Ca^{2+} transients. Ca^{2+} 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^{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^{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^+/Ca^{2+} exchanger(NCX1), and anti-phospho-PLB-S16 from Millipore (Billerica, MA); anti-SERCA2a from Sigma-Aldrich (St.Luis, 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)LVEKVTYLKKCONH2 (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^{2+} alternans was compared using Fisher's exact test. A P value of <0.05 was considered significant.

Results

Dietary n-3 PUFAs do not stabilize intracellular Ca^{2+} cycling in VF+ myocytes and increase susceptibility of VF- myocytes to pro-arrhythmic diastolic Ca^{2+} waves

Recordings of cytosolic Ca^{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 PUFAs when compared to placebo-treated cells (Figure 1 E).

Dietary n-3 PUFAs increase susceptibility of VF-myocytes to pro-arrhythmic Ca^{2+} alternans

To investigate whether the effects of dietary n-3 PUFAs on VF- myocytes were associated with Ca^{2+}-dependent arrhythmogenic substrate, we studied the amplitude and rate-dependence of Ca^{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^{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^{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^{2+} alternans recorded in VF- from n-3 PUFAs treated group at 1 Hz (Figure 2 *B, C, D*). These data suggest that dietary n-3 PUFAs may enhance the dynamic substrate for arrhythmia in VF- hearts.

Effect of dietary n-3 PUFAs on intracellular Ca^{2+} handling in VF- myocytes is associated with the increased ryanodine receptor (RyR2) activity

We further characterized the effect of dietary n-3 PUFAs on properties of intracellular Ca^{2+} handling in VF- myocytes by measuring the frequency of Ca^{2+} sparks. As shown in Figure 3 and Table 1 Ca^{2+} sparks frequency was significantly higher in untreated VF- myocytes when compared to control. However, even greater increases in Ca^{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^{2+} spark activity in VF- myocytes, we studied SR Ca^{2+} content ($[Ca^{2+}]_{SR}$) by measuring the amplitude of Ca^{2+} transients evoked by 10 mM caffeine. As shown in Figure 3 (B and D), $[Ca^{2+}]_{SR}$ was significantly lower in n-3 PUFA-treated VF- myocytes compared to untreated control and VF- myocytes, respectively. More frequent Ca^{2+} sparks at lower $[Ca^{2+}]_{SR}$ 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^{2+} cycling occur following chronic dietary supplementation with n-3 PUFAs. Dietary n-3 PUFAs did not significantly affect expression of RyR2, SR Ca^{2+} ATPase, phospholamban (PLB), alpha subunit of cardiac L-type Ca^{2+} channels, and Na^+/Ca^{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).
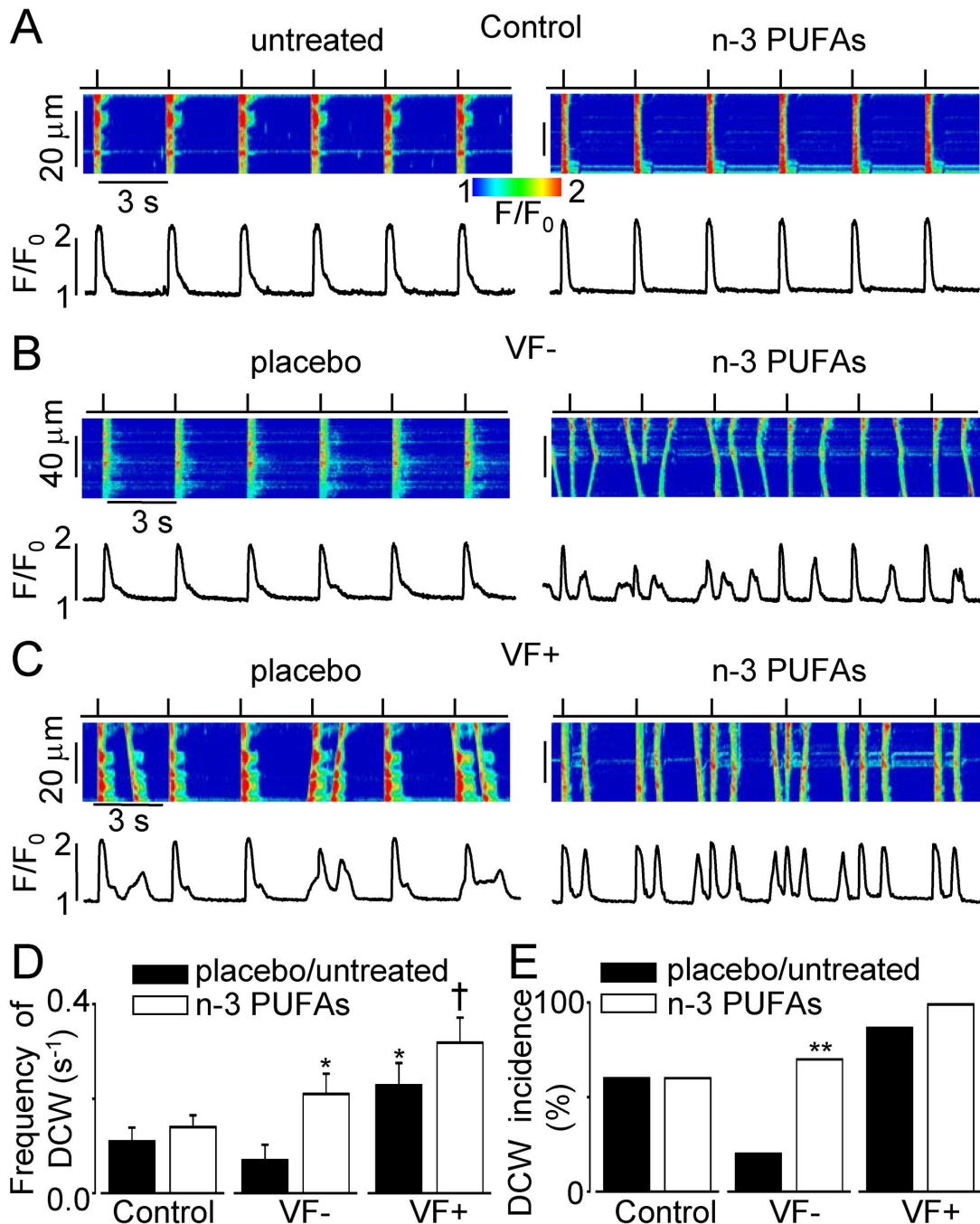
Figure 1. Dietary n-3 PUFAs induce pro-arrhythmic remodeling of intracellular Ca²⁺ handling in VF-myocytes. Representative line-scan images and corresponding profiles of Rhod-2 fluorescence during periodic (0.3 Hz) electrical stimulation recorded in myocytes from placebo/untreated and n-3 PUFA-treated 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 14 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.

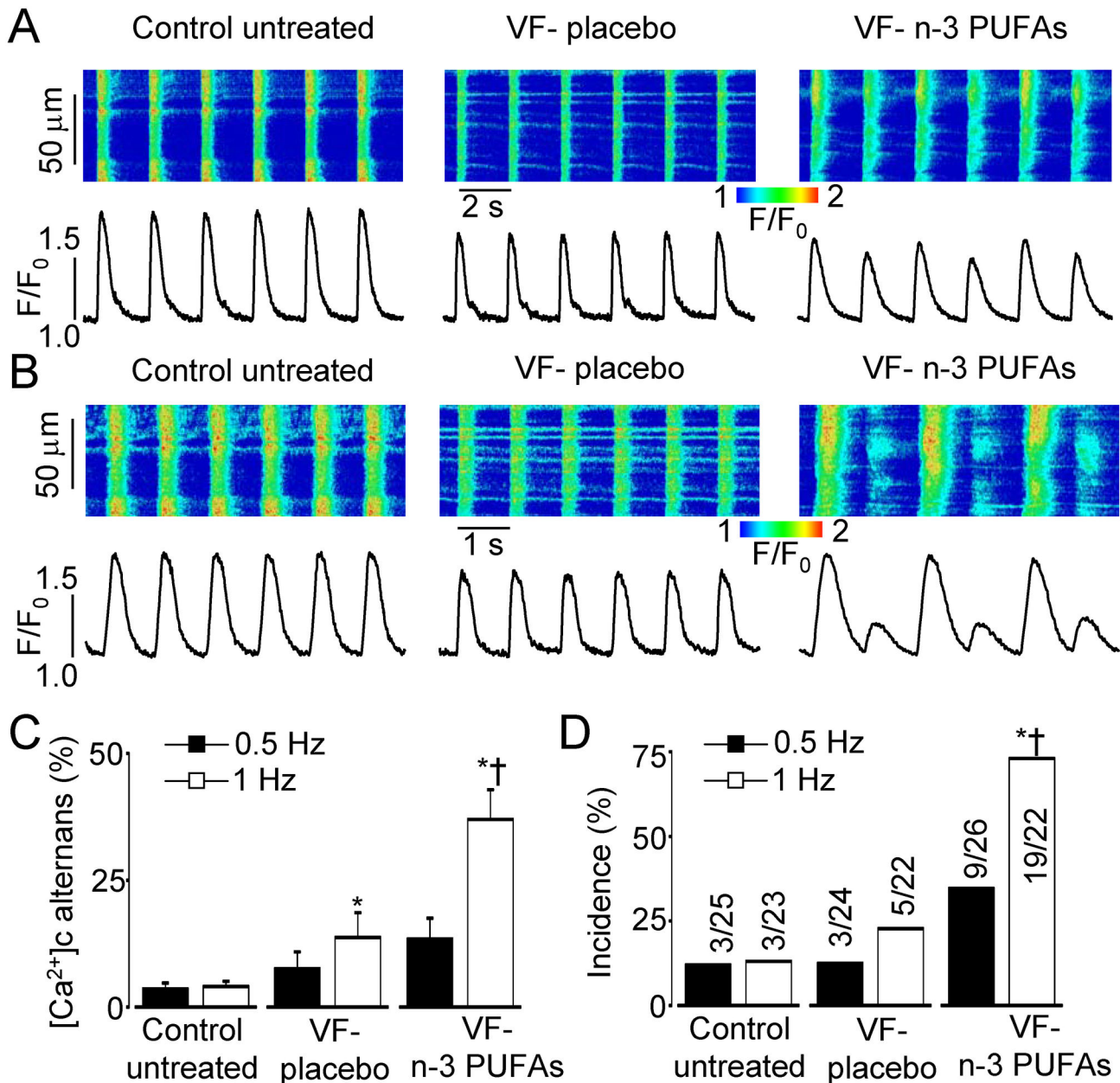doi: 10.1371/journal.pone.0078414.g001

Figure 2. Dietary n-3 PUFAs increase susceptibility of VF- myocytes to pro-arrhythmic Ca^{2+} alternans. Representative line-scan images and corresponding profiles of Rhod-2 fluorescence recorded in myocytes from indicated groups at 0.5 (**A**) and 1 Hz (**B**) stimulation. Bar graphs show amplitude (**C**) and incidence (**D**) of Ca^{2+} alternans recorded in control untreated myocytes and VF-myocytes from placebo and n-3 PUFAs groups at 0.5 and 1Hz. Number of myocytes with amplitude of Ca^{2+} alternans larger than 10 % (numerator) and total number of myocytes studied (denominator) are indicated for each group presented in panel D bar graph. *, P<0.05 *vs.* control (1Hz); †, P<0.05 *vs.* VF- placebo (1Hz).

doi: 10.1371/journal.pone.0078414.g002

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
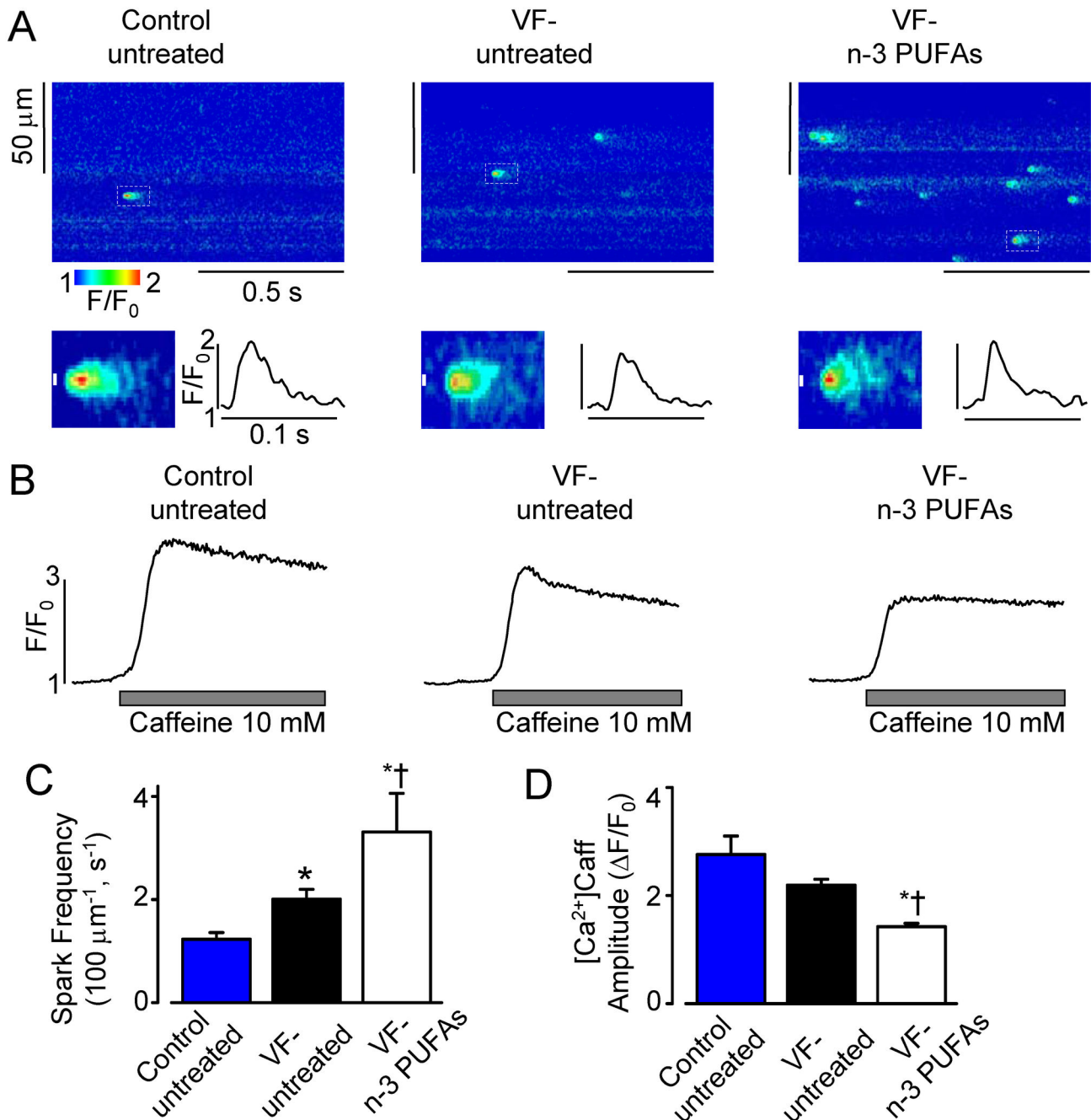
Figure 3. Dietary n-3 PUFAs increase frequency of Ca²⁺ sparks in VF- myocytes. **A**, Representative line-scan images of Ca²⁺ sparks recorded in saponin-permeabilized myocytes from indicated groups. Insets show scaled up image of Ca²⁺ spark with corresponding time-dependent fluorescence profile. **B**, Representative traces of Ca²⁺ transients evoked by 10 mM caffeine recorded in permeabilized myocytes from indicated groups. **C**, Average Ca²⁺ spark frequency (in 100 $\mu m^{-1}*s^{-1}$) 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 Ca²⁺ transients ([Ca²⁺]$_{CAFF}$, $\Delta F/F_0$) 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.

doi: 10.1371/journal.pone.0078414.g003

Table 1. Properties of Ca^{2+} sparks in saponin-permeabilized ventricular myocytes.

	Control untreated $n=52$	VF- untreated $n=47$	VF- n-3 PUFAs $n=45$
Amplitude ($\Delta F/F_0$)	0.74±0.02	0.64±0.01*	0.70±0.01[†]
Frequency (sparks/100 μm/s)	1.23±0.13	2.01±0.19*	3.31±0.38*[†]
FDHM (ms)	21.9±0.8	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.

doi: 10.1371/journal.pone.0078414.t001

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 Ca^{2+} dysregulation is recognized as an important factor contributing to the genesis of various forms of cardiac arrhythmias. Remodeling of intracellular Ca^{2+} cycling leading to increased occurrences of spontaneous Ca^{2+} releases and diastolic Ca^{2+} waves is typically associated with triggered arrhythmias [10,11,13]. Alterations in intracellular Ca^{2+} handling resulting in beat-to-beat variations in the amplitude of Ca^{2+} transient (Ca^{2+} 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 [34] and Ca^{2+} alternans [31] when compared to myocytes isolated from normal hearts. In the present study using the same animal model, we investigated the effects of dietary n-3 PUFAs on intracellular Ca^{2+} cycling [23]. Dietary EPA +DHA ethyl esters supplements significantly increased left ventricular n-3 PUFA content [7,27]. The increased n-3 PUFA tissue content did not alter the already high propensity of VF+ myocytes for DCWs (Figure 1 C, D). Furthermore dietary n-3 PUFAs increased susceptibility of VF- myocytes to both DCWs and Ca^{2+} alternans (Figure 1 *A, B*, *D* and Figure 2 B-D). Although molecular mechanisms responsible for these effects of n-3 PUFAs remain to be determined, our cellular data demonstrate that incorporated n-3 PUFAs can be linked to increased susceptibility to both triggered and reentrant arrhythmias in post-MI hearts.

It has been previously noted that the physiological effects of n-3 PUFAs might depend on the route of administration: acute application of free n-3 PUFAs vs. chronic dietary consumption 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 Ca^{2+} 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].

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 Ca^{2+} transients under baseline conditions, but reduced both arrhythmogenic Ca^{2+} after-transients and arrhythmic contractile activity evoked by beta-adrenergic receptor stimulation [21,22]. We previously showed that incorporated n-3 PUFAs did not change Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 PUFAs 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 PUFAs alter expression levels of proteins involved in cardiac Ca^{2+} cycling including RyR2 (Figure 4, table 2). Since phosphorylation of RyR2 has been implicated in abnormal increase of RyR2 activity in disease states [32,33] and acute application of n-3 PUFAs has been associated with activation of protein kinase A [39], we also studied the effects of dietary n-3 PUFAs on phosphorylation state of RyR2. As illustrated in Figure 4 (B) and table 2, phosphorylation of established RyR2 phosphorylation sites was not significantly altered by dietary n-3 PUFAs suggesting that proarrhythmic effects of dietary n-3 PUFAs are not associated with the increased RyR2 phosphorylation. Further research will be needed to determine molecular mechanisms linking dietary n-3 PUFA and abnormal RyR2 activity.

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
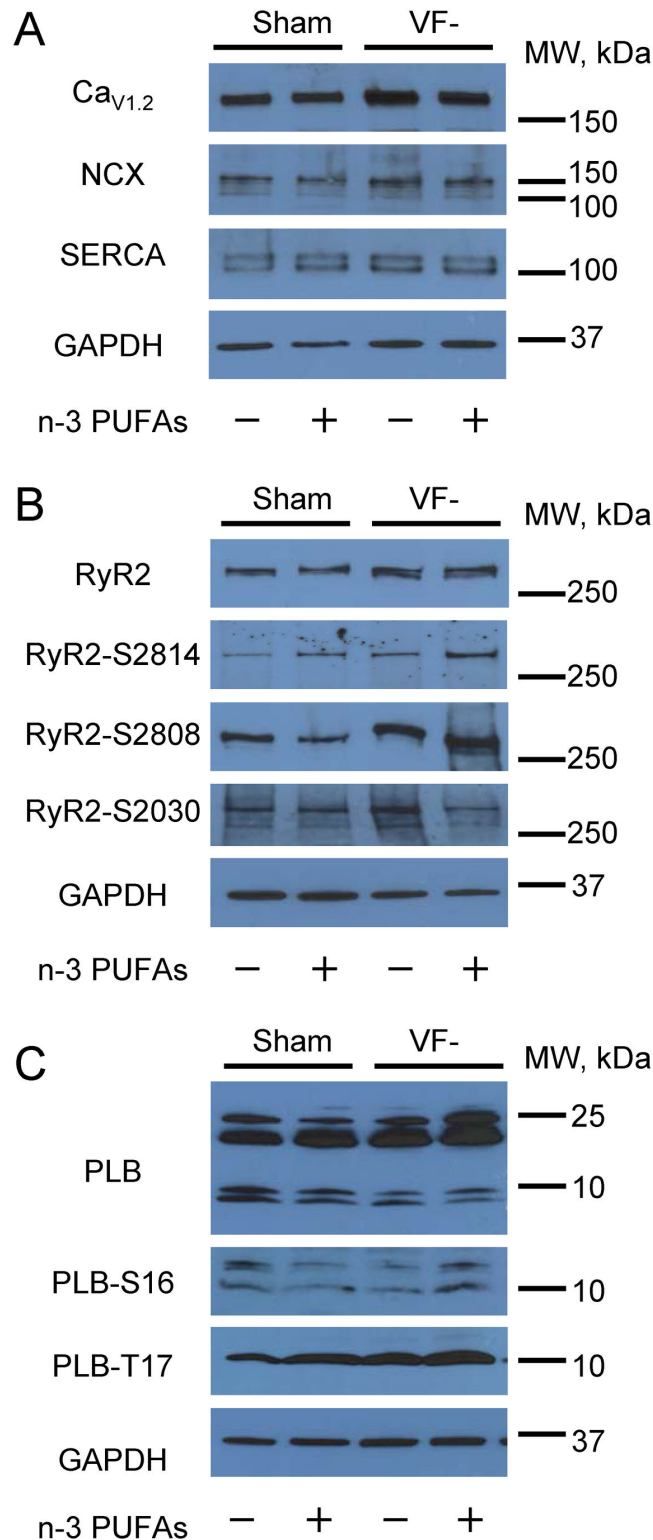
Figure 4. Dietary n-3 PUFAs do not affect expression and phosphorylation levels of proteins involved in cardiac Ca²⁺ cycling. A-C, Representative immunoblots of left ventricle homogenates prepared from placebo and n-3 PUFA-treated control (sham) and VF- groups. NCX1, Na⁺/Ca²⁺ exchanger type 1; SERCA2a, cardiac isoform of SR Ca²⁺-ATPase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; PLN, phospholamban; Cav1.2, α1C subunit of L-type Ca²⁺ channel. Data were obtained using 2-4 heart samples. Quantitative analysis is presented in Table 2.

Table 2. Expression and phosphorylation levels of critical Ca^{2+} handling proteins in n-3 PUFA-treated control and VF-preparations.

	Control n-3 PUFAs (Normalized to control placebo, %)	VF- n-3 PUFAs (Normalized to VF- placebo, %)
RyR2	132±18	116±34
RyR2-S2808	75±7	115±27
RyR2-S2814	180±47	97±29
RyR2-S2030	111±26	95±22
SERCA2A	98±35	149±29
PLB	90±7	88±11
PLB-S16	125±49	365±72
PLB-T17	158±19	125±12
Cav1.2	135±33	145±49
NCX	87±20	139±53

Data were obtained from 2-4 experiments. No significant differences (P>0.05) were observed in protein expression or protein phosphorylation levels between placebo and n-3 PUFAs treatments.

doi: 10.1371/journal.pone.0078414.t002

room temperature (22-24°C) and ambient O_2 tension (~20%) in contrast to physiological temperature (37°C) and O_2 tension (~5%). Therefore, our experimental conditions could influence dynamics of intracellular Ca^{2+} 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.

References

1. Daviglus ML, Stamler J, Orencia; Daviglus ML, Stamler J, Orencia AJ, Dyer AR, Liu K, Greenland P, Walsh MK, Morris D, Shekelle RBAJ, Dyer AR, Liu K, et al (1997) Fish consumption and the 30-year risk of fatal myocardial infarction. N Engl J Med 336: 1046-1053. doi:10.1056/NEJM199704103361502. PubMed: 9091800. doi:10.1056/NEJM199704103361502 PubMed: 9091800
2. Albert CM, Hennekens CH, O'Donnell CJ, Ajani UA, Carey VJ et al. (1998) Fish consumption and risk of sudden cardiac death. JAMA 279: 23-28. doi:10.1001/jama.279.1.23. PubMed: 9424039.
3. Mozaffarian D, Lemaitre RN, Kuller LH, Burke GL, Tracy RP et al. (2003) Cardiac benefits of fish consumption may depend on the type of fish meal consumed: the Cardiovascular Health Study. Circulation 107: 1372-1377. doi:10.1161/01.CIR.0000055315.79177.16. PubMed: 12642356.
4. Rizos EC, Ntzani EE, Bika E, Kostapanos MS, Elisaf MS (2012) Association between omega-3 fatty acid supplementation and risk of major cardiovascular disease events: a systematic review and meta-analysis. JAMA 308: 1024-1033. doi:10.1001/2012.jama.11374. PubMed: 22968891.
5. Rauch B, Senges J (2012) The effects of supplementation with omega-3 polyunsaturated Fatty acids on cardiac rhythm: anti-arrhythmic, pro-arrhythmic, both or neither? It depends. Front. Physiol (Bethesda Md.) 3: 57.
6. Den Ruijter HM, Berecki G, Opthof T, Verkerk AO, Zock PL et al. (2007) Pro- and antiarrhythmic properties of a diet rich in fish oil. Cardiovasc Res 73: 316-325. doi:10.1016/j.cardiores.2006.06.014. PubMed: 16859661.
7. Billman GE, Carnes CA, Adamson PB, Vanoli E, Schwartz PJ (2012) Dietary omega-3 fatty acids and susceptibility to ventricular fibrillation: lack of protection and a proarrhythmic effect. Circ Arrhythm. J Electrophysiol 5: 553-560.
8. Richardson ES, Iaizzo PA, Xiao YF (2011) Electrophysiological mechanisms of the anti-arrhythmic effects of omega-3 fatty acids. J Cardiovasc Transl Res 4: 42-52 PubMed: 21125434

9. Weiss JN, Karma A, Shiferaw Y, Chen PS, Garfinkel A et al. (2006) From pulsus to pulseless: the saga of cardiac alternans. Circ Res 98: 1244-1253. doi:10.1161/01.RES.0000224540.97431.f0. PubMed: 16728670.
10. Laurita KR, Rosenbaum DS (2008) Mechanisms and potential therapeutic targets for ventricular arrhythmias associated with impaired cardiac calcium cycling. J Mol Cell Cardiol 44: 31-43. doi:10.1016/j.yjmcc.2007.10.012. PubMed: 18061204.
11. Ter Keurs HE, Boyden PA (2007) Calcium and arrhythmogenesis. Physiol Rev 87: 457-506. doi:10.1152/physrev.00011.2006. PubMed: 17429038.
12. Janse MJ, Wit AL (1989) Electrophysiological mechanisms of ventricular arrhythmias resulting from myocardial ischemia and infarction. Physiol Rev 69: 1049-1169. PubMed: 2678165.
13. Pogwizd SM, Bers DM (2004) Cellular basis of triggered arrhythmias in heart failure. Trends Cardiovasc Med 14: 61-66. doi:10.1016/j.tcm.2003.12.002. PubMed: 15030791.
14. Honen BN, Saint DA, Laver DR (2003) Suppression of calcium sparks in rat ventricular myocytes and direct inhibition of sheep cardiac RyR channels by EPA, DHA and oleic acid. J Membr Biol 196: 95-103. doi:10.1007/s00232-003-0628-9. PubMed: 14724746.
15. Den Ruijter HM, Berecki G, Verkerk AO, Bakker D, Baartscheer A et al. (2008) Acute administration of fish oil inhibits triggered activity in isolated myocytes from rabbits and patients with heart failure. Circulation 117: 536-544. doi:10.1161/CIRCULATIONAHA.107.733329. PubMed: 18195172.
16. Rodrigo GC, Dhanapala S, Macknight AD (1999) Effects of eicosapentaenoic acid on the contraction of intact, and spontaneous contraction of chemically permeabilized mammalian ventricular myocytes. J Mol Cell Cardiol 31: 733-743. doi:10.1006/jmcc.1998.0914. PubMed: 10329201.
17. Xiao YF, Gomez AM, Morgan JP, Lederer WJ, Leaf A (1997) Suppression of voltage-gated L-type Ca^{2+} currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. Proc Natl

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 Ca^{2+} sparks in myocytes from post-MI (VF-) hearts. The increased frequency of Ca^{2+} sparks along with the reduced SR Ca^{2+} content observed in VF- myocytes suggest that incorporated n-3 PUFAs increased sensitivity of ryanodine receptors to SR Ca^{2+} 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 Ca^{2+} 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.

Acad Sci U S A 94: 4182-4187. doi:10.1073/pnas.94.8.4182. PubMed: 9108126.

18. Ferrier GR, Redondo I, Zhu J, Murphy MG (2002) Differential effects of docosahexaenoic acid on contractions and L-type Ca²⁺ current in adult cardiac myocytes. Cardiovasc Res 54: 601-610. doi:10.1016/S0008-6363(02)00275-4. PubMed: 12031706.

19. Negretti N, Perez MR, Walker D, O'Neill SC (2000) Inhibition of sarcoplasmic reticulum function by polyunsaturated fatty acids in intact, isolated myocytes from rat ventricular muscle. J Physiol 523 2: 367-375. doi:10.1111/j.1469-7793.2000.t01-1-00367.x. PubMed: 10699081.

20. Swan JS, Dibb K, Negretti N, O'Neill SC, Sitsapesan R (2003) Effects of eicosapentaenoic acid on cardiac SR Ca²⁺-release and ryanodine receptor function. Cardiovasc Res 60: 337-346. doi:10.1016/S0008-6363(03)00545-5. PubMed: 14613863.

21. Leifert WR, Dorian CL, Jahangiri A, McMurchie EJ (2001) Dietary fish oil prevents asynchronous contractility and alters Ca²⁺ handling in adult rat cardiomyocytes. J Nutr Biochem 12: 365-376. doi:10.1016/S0955-2863(01)00151-6. PubMed: 11516641.

22. Berecki G, Den Ruijter HM, Verkerk AO, Schumacher CA, Baartscheer A et al. (2007) Dietary fish oil reduces the incidence of triggered arrhythmias in pig ventricular myocytes. Heart Rhythm 4: 1452-1460. doi:10.1016/j.hrthm.2007.07.015. PubMed: 17954406.

23. Billman GE (2006) A comprehensive review and analysis of 25 years of data from an in vivo canine model of sudden cardiac death: implications for future anti-arrhythmic drug development. Pharmacol Ther 111: 808-835. doi:10.1016/j.pharmthera.2006.01.002. PubMed: 16483666.

24. Billman GE, Schwartz PJ, Stone HL (1982) Baroreceptor reflex control of heart rate: a predictor of sudden cardiac death. Circulation 66: 874-880. doi:10.1161/01.CIR.66.4.874. PubMed: 7116603.

25. Schwartz PJ, Billman GE, Stone HL (1984) Autonomic mechanisms in ventricular fibrillation induced by myocardial ischemia during exercise in dogs with healed myocardial infarction. An experimental preparation for sudden cardiac death. Circulation 69: 790-800. doi:10.1161/01.CIR.69.4.790. PubMed: 6697463.

26. Di Stasi D, Bernasconi R, Marchioli R, Marfisi RM, Rossi G et al. (2004) Early modifications of fatty acid composition in plasma phospholipids, platelets and mononucleates of healthy volunteers after low doses of n-3 polyunsaturated fatty acids. Eur J Clin Pharmacol 60: 183-190. doi:10.1007/s00228-004-0758-8. PubMed: 15069592.

27. Billman GE, Nishijima Y, Belevych AE, Terentyev D, Xu Y et al. (2010) Effects of dietary omega-3 fatty acids on ventricular function in dogs with healed myocardial infarctions: in vivo and in vitro studies. Am J Physiol Heart Circ Physiol 298: H1219-H1228. doi:10.1152/ajpheart.01065.2009. PubMed: 20097770.

28. Sridhar A, Nishijima Y, Terentyev D, Terentyeva R, Uelmen R et al. (2008) Repolarization abnormalities and afterdepolarizations in a canine model of sudden cardiac death. Am J Physiol Regul Integr Comp Physiol 295: R1463-R1472. doi:10.1152/ajpregu.90583.2008. PubMed: 18768760.

29. Cheng H, Song LS, Shirokova N, González A, Lakatta EG et al. (1999) Amplitude distribution of calcium sparks in confocal images: theory and studies with an automatic detection method. Biophys J 76: 606-617. doi:10.1016/S0006-3495(99)77229-2. PubMed: 9929467.

30. Belevych AE, Sansom SE, Terentyeva R, Ho HT, Nishijima Y et al. (2011) MicroRNA-1 and -133 increase arrhythmogenesis in heart failure by dissociating phosphatase activity from RyR2 complex. PLOS ONE 6: e28324. doi:10.1371/journal.pone.0028324. PubMed: 22163007.

31. Belevych AE, Terentyev D, Viatchenko-Karpinski S, Terentyeva R, Sridhar A et al. (2009) Redox modification of ryanodine receptors underlies calcium alternans in a canine model of sudden cardiac death. Cardiovasc Res 84: 387-395. doi:10.1093/cvr/cvp246. PubMed: 19617226.

32. Belevych AE, Radwański PB, Carnes CA, Györke S (2013) 'Ryanopathy': causes and manifestations of RyR2 dysfunction in heart failure. Cardiovasc Res 98: 240-247. doi:10.1093/cvr/cvt024. PubMed: 23408344.

33. Niggli E, Ullrich ND, Gutierrez D, Kyrychenko S, Poláková E et al. (2012) Posttranslational modifications of cardiac ryanodine receptors: Ca²⁺ signaling and EC-coupling. Biochim Biophys Acta 1833: 866-875. PubMed: 22960642.

34. Belevych AE, Terentyev D, Terentyeva R, Ho HT, Gyorke I et al. (2012) Shortened Ca²⁺ signaling refractoriness underlies cellular arrhythmogenesis in a postinfarction model of sudden cardiac death. Circ Res 110: 569-577. doi:10.1161/CIRCRESAHA.111.260455. PubMed: 22223353.

35. London B, Albert C, Anderson ME, Giles WR, Van Wagoner DR et al. (2007) Omega-3 fatty acids and cardiac arrhythmias: prior studies and recommendations for future research: a report from the National Heart, Lung, and Blood Institute and Office Of Dietary Supplements Omega-3 Fatty Acids and their Role in Cardiac Arrhythmogenesis Workshop. Circulation 116: e320-e335. doi:10.1161/CIRCULATIONAHA.107.712984. PubMed: 17768297.

36. Billman GE, Hallaq H, Leaf A (1994) Prevention of ischemia-induced ventricular fibrillation by omega 3 fatty acids. Proc Natl Acad Sci U S A 91: 4427-4430. doi:10.1073/pnas.91.10.4427. PubMed: 8183925.

37. Abdukeyum GG, Owen AJ, McLennan PL (2008) Dietary (n-3) long-chain polyunsaturated fatty acids inhibit ischemia and reperfusion arrhythmias and infarction in rat heart not enhanced by ischemic preconditioning. J Nutr 138: 1902-1909. PubMed: 18806099.

38. Coronel R, Wilms-Schopman FJ, Den Ruijter HM, Belterman CN, Schumacher CA et al. (2007) Dietary n-3 fatty acids promote arrhythmias during acute regional myocardial ischemia in isolated pig hearts. Cardiovasc Res 73: 386-394. doi:10.1016/j.cardiores.2006.10.006. PubMed: 17116294.

39. Szentandrássy N, Pérez-Bido MR, Alonzo E, Negretti N, O'Neill SC (2007) Protein kinase A is activated by the n-3 polyunsaturated fatty acid eicosapentaenoic acid in rat ventricular muscle. J Physiol 582: 349-358. doi:10.1113/jphysiol.2007.132753. PubMed: 17510185.

40. Harris WS, Sands SA, Windsor SL, Ali HA, Stevens TL et al. (2004) Omega-3 fatty acids in cardiac biopsies from heart transplantation patients: correlation with erythrocytes and response to supplementation. Circulation 110: 1645-1649. doi:10.1161/01.CIR.0000142292.10048.B2. PubMed: 15353491.