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RESEARCH TOPICS

THE EFFECTS OF OMEGA-3 POLYUNSATURATED FATTY ACIDS ON CARDIAC RHYTHM: ANTIARRHYTHMIC, PROARRHYTHMIC, BOTH OR NEITHER?

Hosted by
George E. Billman

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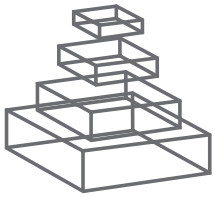

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THE EFFECTS OF OMEGA-3 POLYUNSATURATED FATTY ACIDS ON CARDIAC RHYTHM: ANTIARRHYTHMIC, PROARRHYTHMIC, BOTH OR NEITHER?

Hosted By:
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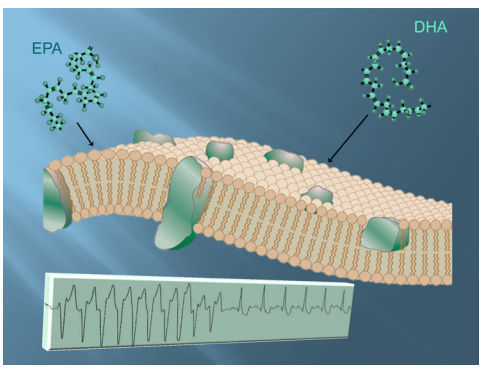

Interaction of omega-3 polyunsaturated fatty acids with the cardiac cell membrane and their possible effects on ventricular arrhythmias.
Image by George E. Billman.

The cardiovascular benefits of dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) have been actively investigated for nearly 40 years. Beginning with the pioneering studies of Bang and Dyerberg, epidemiological data provide strong evidence for an inverse relationship between fatty fish consumption and cardiac mortality. In contrast to these observational studies, interventional studies using n-3 PUFAs for the secondary prevention of adverse cardiovascular events in patients with heart disease have yielded conflicting results; some studies have reported reduced sudden cardiac death or mortality, while other more recent studies have reported either no effect or an increase in adverse cardiac events. Nevertheless, the American Heart Association and the American College of Cardiology continue to recommend fish oils for the secondary prevention of coronary artery disease. Based in part upon these recommendations, consumer demand for n-3 PUFA products (both nutritional supplements and foods enriched with these lipids) has exploded. In the United States alone, it has been estimated that in 2004, 5-10% of the adult U.S. population were taking a fish oil supplement, with annual sales growth of 40%. In fact, the sales of these products are projected to exceed 7 billion dollars by the end of 2011 (www.marketresearch.com, product reports).

Despite the extensive marketing of fish oil products, a scientific consensus on the effects of n-3 PUFA on cardiac rhythm has yet to be reached. It is the purpose of this Research Topic to stimulate a discussion on the putative benefits of n-3 PUFAs on cardiac rhythm. Authors are invited to submit clinical, translational, or experimental research articles, reviews, and hypotheses that address the actions of n-3 PUFA (positive, negative, or neutral) on cardiac rhythm and cardiac electrophysiology. Studies that evaluate the effects of n-3 PUFA on myocyte electrical properties, atrial fibrillation, ventricular fibrillation, and heart rate variability are particularly welcome.

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Omega-3 polyunsaturated fatty acids and cardiac rhythm: an introduction

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"Sine doctrina, vita est quasi mortis imago" [Without, education, life is but the image of death] Dionysius Cato (Roman author, Fl. 4th c. AD).

"If I can stop one heart from breaking, I shall not live in vain" Emily Dickinson (American poet, 1830–1886).

The effective management of cardiac arrhythmias, either of atrial or of ventricular origin, remains a major challenge for the cardiologist. Sudden cardiac death most frequently due to ventricular tachyarrhythmias (Hinkle and Thaler, 1982; Bayes de Luna et al., 1989; Greene, 1990) remains the leading cause of death in industrially developed countries, accounting for between 300,000 and 500,000 deaths each year in the United States (Abildstrom et al., 1999; Zheng et al., 2001). In a similar manner, atrial fibrillation is the most common rhythm disorder (Kannel et al., 1998; Lakshminarayan et al., 2006), accounting for about 2.3 million cases in the United States and has been projected to increase by 2.5-fold over the next half century (Anonymous, 1998). Indeed, the prevalence of this arrhythmia increases with each decade of life (0.5% patient population between the ages of 50 and 59 years climbing to almost 9% at age 80–89 years) and contributes to approximately one-quarter of ischemic strokes in the elderly population (Kannel et al., 1998; Lakshminarayan et al., 2006). The economic impact associated with the morbidity and mortality resulting from cardiac arrhythmias is enormous [incremental cost per quality-adjusted life-year as much as US $558,000 (Byrant et al., 2005)].

Despite the enormity of this problem, the development of safe and effective anti-arrhythmic agents remains elusive. Several anti-arrhythmic drugs have actually been shown to increase, rather than to decrease, the risk for arrhythmic death in patients recovering from myocardial infarction (Echt et al., 1991; Waldo et al., 1996) while even "optimal" pharmacological therapy fails to suppress these arrhythmias completely (Buxton et al., 1999). For example, the one-year mortality is 10% or higher, with sudden death accounting for approximately one-third of the deaths, in post-myocardial infarction patients treated with β-adrenergic receptor antagonists (Buxton et al., 1999). Implantable cardioverter defibrillators (ICDs) have been shown to reduce cardiac mortality, providing a better protection from sudden death than current pharmacological therapy in certain high-risk patient populations (Buxton et al., 1999; Connelly et al., 2000). However, these devices are expensive to use and maintain (Groeneveld et al., 2006), negatively affect the patient's quality of life (Groeneveld et al., 2006), have a significant risk for inappropriate shock delivery (Poole et al., 2008), are ineffective in females patients (Henyan et al., 2006), and, perhaps most importantly, only extend life by a mean of 4.4 months (Connelly et al., 2000). Given the adverse outcomes associated with ICDs and many anti-arrhythmic medications, as well as the partial protection afforded by even the best agents (e.g., β-adrenergic receptor antagonists and ICDs), it is obvious that more effective anti-arrrhythmic therapies must be developed.

The cardiovascular benefits of dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) have been actively investigated for nearly 40 years. Beginning with the pioneering studies of Bang and Dyerberg (Dyerberg et al., 1978; Bang et al., 1980), epidemiological data provide strong evidence for an inverse relationship between fatty fish consumption and cardiac mortality (Kromhout et al., 1985; Daviglus et al., 1997). In contrast to these observational studies, interventional studies using n-3 PUFAs for the secondary prevention of adverse cardiovascular events in patients with heart disease have yielded conflicting results. Some studies have reported reduced sudden cardiac death or mortality (Burr et al., 1989; Marchioli et al., 2002), while other more recent studies have reported that n-3 PUFAs either had no effect on cardiac arrhythmias [either ventricular arrhythmias/sudden death (Brouwer et al., 2006; Yokoyama et al., 2007; GISSI-HF Investigators, 2008; Kromhout et al., 2010; Rauch et al., 2010) or atrial fibrillation (Kowey et al., 2010; Mozaffarian et al., 2012; Sandesara et al., 2012)] or actually increased adverse cardiac events (Burr et al., 2003; Raitt et al., 2005). Not surprisingly, meta-analysis of these studies have yielded similar conflicting results (Hooper et al., 2004; Jenkins et al., 2008; Brouwer et al., 2009; Leon et al., 2009; Zhao et al., 2009; Filion et al., 2010) with the most recent study finding that omega-3 fatty acids were neutral, neither increasing nor decreasing the risk for arrhythmias (Rizos et al., 2012). Similar conflicting results have been obtained from animals models (McLennan et al., 1988; Billman et al., 1994; Coronel et al., 2007; Billman et al., 2012). Of particular note, dietary n-3 PUFAs increased rather than decreased susceptibility to arrhythmias induced by regional myocardial ischemia in isolated hearts (Coronel et al., 2007) and provoked ventricular fibrillation in conscious animals previously shown to be at a low risk for malignant arrhythmias (Billman et al., 2012). Despite these inconsistent findings, the American Heart Association and the American College of Cardiology continue to recommend fish oils for the secondary prevention of coronary artery disease (Kris-Etherton et al., 2003; Smith et al., 2006). Based in part upon these recommendations, consumer demand for n-3 PUFA products (both nutritional supplements

and foods enriched with these lipids) has exploded. It has been estimated that 5–10% of the adult US population use fish oil supplements and sales are projected to exceed 7 billion dollars by the end of 2011 [www.marketresearch.com, product reports].

Despite the intensive marketing of fish oil products, a scientific consensus on the effects of n-3 PUFA on cardiac rhythm has yet to be reached. It is the purpose of this book to stimulate a discussion on the putative benefits of n-3 PUFAs on cardiac rhythm. The book contains both state-of-the art reviews of the literature and original research articles that address various aspects of the effects of n-3 PUFAs on cardiac rhythm. The book is divided into three sections. The first section addresses the effects of n-3 PUFAs on heart rate variability (chapters 2–4). The second section provides comprehensive reviews of the effects of n-3 PUFAs on ventricular arrhythmias/sudden death (chapters 5–8) and on atrial fibrillation (chapters 8–10). The third and final section (chapters 11–16) evaluates the cellular mechanisms by which n-3 PUFAs can influence arrhythmia formation. By understanding how n-3 PUFAs affect the cardiac rhythm, the author hopes that this brief monograph will provide an education sufficient to keep at least one heart from breaking.

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Omega-3 polyunsaturated fatty acids and heart rate variability

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Omega-3 polyunsaturated fatty acids (PUFA) may modulate autonomic control of the heart because omega-3 PUFA is abundant in the brain and other nervous tissue as well as in cardiac tissue. This might partly explain why omega-3 PUFA offer some protection against sudden cardiac death (SCD). The autonomic nervous system is involved in the pathogenesis of SCD. Heart rate variability (HRV) can be used as a non-invasive marker of cardiac autonomic control and a low HRV is a predictor for SCD and arrhythmic events. Studies on HRV and omega-3 PUFA have been performed in several populations such as patients with ischemic heart disease, patients with diabetes mellitus, patients with chronic renal failure, and in healthy subjects as well as in children. The studies have demonstrated a positive association between cellular content of omega-3 PUFA and HRV and supplementation with omega-3 PUFA seems to increase HRV which could be a possible explanation for decreased risk of arrhythmic events and SCD sometimes observed after omega-3 PUFA supplementation. However, the results are not consistent and further research is needed.

Keywords: omega-3 polyunsaturated fatty acids, sudden cardiac death, autonomic function, heart rate variability

INTRODUCTION

Cardiac autonomic control is important in the pathogenesis of sudden cardiac death (SCD). Increased vagal activity is considered protective against SCD (Billman et al., 1982; Schwartz et al., 1984, 1992; Schwartz, 1998; Airaksinen, 1999) whereas sympathetic activity favors the development of cardiac arrhythmias (Task Force of the European Society of Cardiology, and the North American Society of Pacing and Electrophysiology, 1996). The modulation of autonomic control and a change in vagal tone and/or sympathetic tone may therefore be of major importance for the prevention of SCD (La Rovere et al., 1998).

Possible mechanisms for the protection against SCD from marine omega-3 polyunsaturated fatty acids (PUFA) are a topic in this issue of the Journal. Omega-3 PUFAs are components of membrane phospholipids throughout the body but particular docosahexaenoic acid (DHA) is highly concentrated in the central nervous system where it facilitates neuronal growth and has neuroprotective effects (Beltz et al., 2007; Innis, 2008; Niemoller and Bazan, 2010). As DHA is also abundant in cardiac tissue it is likely that omega-3 PUFA might modulate autonomic control of the heart. This paper reviews the studies on omega-3 PUFA and heart rate variability (HRV) in humans. HRV is a non-invasive marker of cardiac autonomic tone.

HEART RATE VARIABILITY AND HOW CAN IT BE USED?

During sinus rhythm heart rate (HR) and its inverse, the RR-interval, vary from beat-to-beat mainly in response to changes in autonomic function. This beat-to-beat variation termed HRV is a non-invasive method to assess cardiac autonomic tone (Task Force of the European Society of Cardiology, and the North American Society of Pacing and Electrophysiology, 1996). HRV indices (and especially 24-h measurements) seems to be stable and free of placebo effects and HRV indices may thus be a useful tool in assessing the effect of intervention therapies on autonomic function of the heart (Task Force of the European Society of Cardiology, and the North American Society of Pacing and Electrophysiology, 1996).

Heart rate variability can be obtained during a short time period or from 24-h Holter recordings. It can be analyzed in the time domain and frequency domain, or by non-linear methods. Time domain indices (most often used) are based on normal-to-normal beat intervals (RR) but time and frequency domain HRV are closely associated (Kleiger et al., 1991, 2005; Bigger Jr. et al., 1992a).

Two forms of time domain HRV indices are used: (a) data derived directly from the RR interbeat intervals and (b) data derived from differences between successive RR-intervals. Interbeat interval measures are influenced by both short term (e.g., respiratory) and long-term (e.g., circadian) changes (Kleiger et al., 1992). Other time domain indices based on comparisons of lengths of adjacent cycles primarily reflects vagal modulation of the sinoatrial node (Kleiger et al., 1992). Abbreviations of some important time domain HRV indices are listed in **Table 1**.

Studies using frequency domain analyses (Task Force of the European Society of Cardiology, and the North American Society of Pacing and Electrophysiology, 1996; Akselrod et al., 1981; Pomeranz et al., 1985) assessed with spectral analysis have identified a low frequency (LF) band (0.04–0.15 Hz) reflecting both sympathetic and parasympathetic influences (Kingwell et al., 1994;

Abbreviations: CRF, chronic renal failure; DHA, docosahexaenoic acid; DM, diabetes mellitus; EPA, eicosapentaenoic acid; IHD, ischemic heart disease; LF/HF, low frequency band/high-frequency band; MI, myocardial infarction; PUFA, polyunsaturated fatty acids; SCD, sudden cardiac death.

Table 1 | Definition of time domain HRV variables obtained from 24-h Holter recordings.

Variable	Units	Description
RR	ms	Mean of all normal RR-intervals in the 24-h recording
SDNN	ms	SD of all normal RR-intervals in the 24-h recording
SDNN index	ms	Mean of the SD of all normal RR-intervals for all 5-min segments in the 24-h recording
SDANN index	ms	SD of the mean of all normal RR-intervals measured in successive 5-min periods
RMSSD	ms	The square root of the mean of the sum of squares of differences between adjacent RR-intervals in the 24-h recording
pNN50	%	Percentage of successive RR-interval differences >50 ms during the 24-h recording

Modified from Christensen (2003).

Srinivasan et al., 2002; Murray, 2003), and a high-frequency (HF) band (0.15–0.5 Hz), corresponding to respiratory frequency, attributed to parasympathetic influences (Akselrod et al., 1981; Pomeranz et al., 1985; Pagani et al., 1986). The LF/HF ratio has been considered to reflect cardiovascular sympathovagal balance (Pagani et al., 1988; Furlan et al., 1990), but the degree to which this ratio provides a comparison between sympathetic and parasympathetic influences has been questioned (Eckberg, 1997; Karemaker, 1999; Pivik and Dykman, 2004).

An attenuated HRV can reflect an increased sympathetic and/or decreased vagal modulation. These autonomic changes have been associated with an increased risk of malignant ventricular arrhythmias and SCD (Barron and Lesh, 1996; Schwartz, 1998; Airaksinen, 1999). Thus, a question of major importance is whether it is possible to increase HRV and if such an increase would improve clinical outcome. This is not fully answered yet but several pharmacological interventions resulting in an improved patient survival are associated with an increased HRV (Christensen, 2003).

The data from epidemiological and interventional studies on the possible beneficial effect of omega-3 PUFA on SCD makes it of importance whether such an effect can be partly explained by modulation of cardiac autonomic control as evaluated by HRV. Interventional studies on omega-3 PUFA and HRV in humans are summarized in **Table 2**, and the individual studies are dealt with in more detail below.

OMEGA-3 PUFA AND HRV EARLY IN LIFE

The incorporation of omega-3 PUFA in synaptic membranes could potentially influence the autonomic control of the heart. The progressive maturation of the autonomic nervous system during fetal and early life (Massin and von Bernuth, 1997) renders this period a sensitive time, during which supplementation with omega-3 PUFA might exert long-term effects on vagal tone and hence HRV. Studies on HRV and omega-3 supplementation in infants have been performed. In a study by Larnkjaer et al. (2006), no overall effect of omega-3 PUFA supplementation to lactating mothers was found on HRV in the 2.5-year-old offspring. However, in a gender specific analysis, a HRV increasing effect was found in girls.

In another Danish study, 83 healthy infants were randomized to omega-3 PUFA supplementation or no supplementation at 9–12 months of age (Lauritzen et al., 2008). In 57 infants, 0.5-h ECG recordings were successfully obtained before and after the intervention (3 months). Omega-3 PUFA supplementation raised erythrocyte omega-3 PUFA content ($p < 0.001$). An omega-3 PUFA × gender interaction was observed on mean RR-interval ($p = 0.001$) with a 6% longer mean RR-interval in fish-oil-supplemented boys ($p = 0.007$). Irrespective of gender, there was a positive association between the 9- and 12-month changes in RR-interval and erythrocyte omega-3 PUFA ($p < 0.001$). In infants with confirmed changes in erythrocyte omega-3 PUFA, mean RR-interval was found to be longer ($p = 0.011$) in the omega-3 PUFA supplemented group. This study concluded that omega-3 PUFA might affect heart rhythm in infants similar to that observed in some studies with adults.

In a study with a complex design Pivik et al. (2009) studied the effect of early infant diet including omega-3 PUFA on HR and HRV during the first 6 months of life. In the infants fed a DHA-deficient diet, higher HR and lower values for HRV measures were observed and the authors concluded that these findings indicated decreased parasympathetic tone in the DHA-deficient group. These effects appeared at 4 months of age and continued for the remaining 2 months of the study period, and the findings are consistent with suggestions that the 3- to 5-month postnatal interval may be an important period in the development of cardiovascular regulation. It should be emphasized that the acute and long-term clinical consequences of a slightly lower HR or improved HRV in infancy are, however, not known.

OMEGA-3 PUFA AND HRV IN PATIENTS WITH HEART DISEASE

Patients with ischemic heart disease (IHD) are at higher risk of SCD (Zipes and Wellens, 1998), and often have depressed HRV. The association between fish consumption, the content of omega-3 PUFA in cell membranes and HRV was evaluated in 52 patients with a previous MI and a decreased left ventricular ejection fraction (≤0.40; Christensen et al., 1997). Subjects who consumed fish at least once a week had a slightly (non-significant) higher SDNN compared to those never eating fish. These data may be in accordance with the data from Siscovick et al. (2000), and from the US Physicians Health Study (Albert et al., 1998) showing an approximately 50% reduction in the risk of SCD by eating fish once a week, However, these studies included patients without documented IHD although IHD is often the substrate for SCD (Zipes and Wellens, 1998).

It may be the actual membrane level of omega-3 PUFA that determines the susceptibility to develop arrhythmias and SCD (Siscovick et al., 1995; Albert et al., 2002). In the study above (Christensen et al., 1997), the content of omega-3 PUFA was measured in platelets and a close positive association was found between DHA and HRV. Such an association could indicate that supplementation with omega-3 PUFA would increase HRV and this hypothesis was tested in these high-risk patients (Christensen et al., 1996). The subjects were randomized to 5.2 g of omega-3 PUFA daily (8 capsules) for 12 weeks or a comparable amount of olive oil. The HRV parameter SDNN increased significantly from

Table 2 | Interventional studies in humans with omega-3 PUFA and heart rate variability measurements.

Reference	Population	Number (total)	Omega-3 PUFA (daily dose)	Duration of the intervention	HRV indices	Result
Christensen et al. (1996)	Coronary artery disease	49	5.2 g	12 weeks	Time domain (24-h)	+
Christensen et al. (1998)	Dialysis patients	29	5.2 g	12 weeks	Time domain (24-h)	(+)
Christensen et al. (1999)	Healthy	60	2.0 or 6.6 g	12 weeks	Time domain (24-h)	+
Villa et al. (2002)	Coronary heart disease	10	3.0 or 6.0 g	4 weeks	Time and frequency domain (24-h)	(+)
Geelen et al. (2003)	Healthy	84	3.5 g	12 weeks	Time and frequency domain 10 min	−
Dyerberg et al. (2004)	Healthy males			8 weeks	Time domain (24-h)	−
Holguin et al. (2005)	Nursing home residents	52	2.0 g	4 months	6 min repeated Time and frequency domain	+
Romieu et al. (2005)	Nursing home residents	50	2.0 g	5 months	6 min repeated Time and frequency domain	+
O'Keefe et al. (2006)	Coronary artery disease	18	1.5 g	2 × 4 months (cross-over)	76 min Time and frequency domain	+
Hamaad et al. (2006)	Coronary artery disease	38	1.0 g	12 weeks	76 min Time and frequency domain	−
Larnkjaer et al. (2006)	Infants (2.5 years of age)	69	4.5 g	Given to the mothers during 4 months of lactation. The infants were then examined at 2.5 years of age	30 min Time domain	(+)
Svensson et al. (2007)	Dialysis patients	30	1.7 g	12 weeks	24 h Time domain	−
Santini et al. (2007)	Type 2 diabetes	15	1.0 g	6 months	24 h Frequency domain	(+)
Lauritzen et al. (2008)	Infants (9–12 months of age)	83	0.9 g	3 months	30 min Time domain	(+)
Ninio et al. (2008)	Overweight adults	65	6.0 g	12 weeks	20 min Frequency domain	+
DeGiorgio et al. (2008)	Patients with epilepsy	11	2.9 g	12 weeks (cross-over)	60 min Time domain	(+)
Pivik et al. (2009)	Infants (0–6 months)	102	Unknown	6 months	5 min (repeated) Frequency domain	(+)
Carney et al. (2010)	Depressed patients with coronary artery disease	37	2.0 g	10 weeks	24 h Frequency domain	+
Hansen et al. (2010)	Prison inmates	13	1.7 g	23 weeks	5 min Frequency domain	+
Kim et al. (2011)	Patients with mixed dyslipidemia	62	4.0 g	6 weeks	24 h Time and frequency domain	−

A + in the Result column indicates a beneficial effect on HRV, a (+) indicates that subanalysis of the data revealed a beneficial effect on HRV, and − indicates no effect on HRV.

115 to 124 ms in the omega-3 PUFA group. Thus, this was the first study indicating that omega-3 PUFA could beneficially modulate cardiac autonomic control in IHD patients. Another study, by Russo et al. (1995) published 1 year earlier, has in previous papers been referred to as having measured HRV in hypertensive patients given omega-3 PUFA or placebo. They did not Holter monitor the patients nor did they obtain an ECG in these patients. However, they measured HR from ambulatory blood pressure recordings which is equal to HR measured four times per hour during the day and two times per hour during the night. They then reported the SD from these spot measurements as HRV. Thus, this interesting paper did not report HRV according to the usual definition where the beat-to-beat variation obtained in a continuous ECG recording is termed HRV.

Ischemic heart disease is the predominant underlying disease behind SCD (Zipes and Wellens, 1998). HRV was measured in 291 patients referred for coronary angiography due to suspected IHD and these measures were related to cell membrane and adipose tissue levels of omega-3 PUFA (Christensen et al., 2001a). Significant positive correlations were found between time domain HRV indices and levels of omega-3 PUFA, especially DHA. These associations remained significant also after controlling for several possible confounders.

In a smaller cross-over trial in 10 patients with IHD, HRV, and DHA correlated positively after dietary supplementation with omega-3 PUFA (Villa et al., 2002). A decrease in the low frequency band/high-frequency band (LF/HF) was found after having supplemented these patients with 6 g of omega-3 PUFA for 4 weeks. A

low LF/HF is considered to reflect a favorable vagal predominance thus suggesting a protection against SCD.

O'Keefe Jr. et al. (2006) randomized 18 men with a history of MI and ejection fractions <40% to omega-3 PUFA (585 mg of DHA and 225 mg of EPA) or placebo for two 4-month periods in a cross-over design. At the end of each period, HR, HRV, and the rate of HR recovery after exercise were determined. Omega-3 PUFA supplementation decreased HR at rest and increased HRV only in the high-frequency (HF) band. Thus, these changes may also be consistent with an increase in vagal activity.

A smaller single blinded study in 38 post-MI patients using 20-min ECG recordings did not show any changes in HRV indices in the time domain nor in the frequency domain after 3 months of supplementation with omega-3 PUFA or usual care (Hamaad et al., 2006). Nearly 70% of these patients were on beta-adrenergic receptor blockers and/or ACE-inhibitor therapy, agents known to improve HRV. Furthermore, high-risk patients with ventricular ejection fractions <40% and probably a low HRV, were excluded from this study. It is possible that the patients from this study would be the ones who would benefit most from omega-3 PUFA supplementation. The incorporation of omega-3 PUFA in phospholipids were not measured in this study and rather surprisingly the authors did not find any effect of omega-3 PUFA on serum triglycerides in these patients. A triglyceride lowering effect of omega-3 PUFA is one of the most consistent findings (Eslick et al., 2009) and in general such a finding indicates good compliance.

Nodari et al. (2009) investigated the effect of omega-3 PUFA on HRV in 44 patients with idiopathic dilated cardiomyopathy. These patients were without documented IHD. They were randomized to 1 g capsules of omega-3 PUFA or olive oil capsules for 6 months. Compliance was monitored by measuring the plasma levels of omega-3 PUFA before and after intervention. Frequency domain HRV indices were measured and the LF/HF ratio showed a 55% decrease in the treatment group versus a 54% increase in the placebo group. Thus, these results indicated a favorable shift in the cardiac autonomic balance.

Low intake of omega-3 PUFA is associated with depression and low HRV. Carney et al. (2010) therefore examined the effect of omega-3 PUFA on HRV in depressed patients with coronary heart disease. They randomized 72 patients to 2 g of omega-3 PUFA daily or placebo on top of their antidepressive medication (sertraline) for 10 weeks. They measured the frequency domain HRV parameter very low frequency (VLF) before and after supplementation. VLF is strongly correlated to arrhythmic death after an acute MI (Bigger Jr. et al., 1992b). VLF did not change over time in the omega-3 PUFA group but decreased in the placebo group which led the authors to suggest that omega-3 PUFA may have prevented or slowed deterioration in cardiac autonomic function in these high-risk patients.

OMEGA-3 PUFA AND HEART RATE VARIABILITY IN OTHER POPULATIONS

DIABETES MELLITUS

The Framingham study was the first epidemiological report demonstrating a two to fourfold increased risk of angina pectoris and acute MI in diabetes mellitus (DM) patients (Kannel and McGee, 1979). These patients also have an excess post-MI mortality (Mak and Topol, 2000). Autonomic neuropathy involving the heart may be of importance and cardiac autonomic neuropathy carries an excess risk of mortality in patients with DM, including a high risk of SCD (Maser and Lenhard, 2005). HRV analysis is a well established tool in the early detection of autonomic neuropathy in patients with DM (Stein and Kleiger, 1999).

Heart rate variability was examined in 43 type 1 and 38 type 2 diabetes patients and related to omega-3 PUFA content in platelet membranes (Christensen et al., 2001b). In type 1 DM patients HRV increased with increasing levels of DHA. Furthermore, this positive correlation between HRV and platelet DHA was more pronounced in patients with type 1 DM solely receiving insulin therapy and without signs of diabetic complications. However, this study could not demonstrate a significant association between omega-3 PUFA and HRV in the patients with type 2 DM. In contrast, a small Italian study found that 6 months of omega-3 PUFA treatment in a group of 13 type 2 DM patients partially improved HRV in the frequency domain (Santini et al., 2007).

Overweight persons with an increased risk of type 2 DM have an impaired HRV (Ninio et al., 2008). In a randomized, double-blind, parallel comparison, 65 overweight volunteers consumed DHA 1.56 g/day and EPA 0.36 g/day or sunflower-seed oil (placebo) for 12 weeks (Ninio et al., 2008). In 46 of these subjects HRV was assessed in the frequency domain using 20 min ECG recordings. Omega-3 PUFA supplementation improved HRV by increasing HF power, representing parasympathetic activity, and it also reduced HR at rest and during submaximal exercise. Thus, the authors concluded that dietary supplementation with DHA-rich fish oil reduced HR and modulated HRV in a favorable way in these overweight subjects with a high risk of IHD.

CHRONIC RENAL FAILURE

Approximately 50% of the mortality in patients with chronic renal failure (CRF) receiving dialysis is due to cardiovascular disease and it is estimated that SCD accounts for approximately 30% of total mortality in patients with end stage renal disease (ESRD; Herzog et al., 2008). Cardiac autonomic dysfunction is very frequent in these patients (Barron and Lesh, 1996) with a high prevalence of ventricular arrhythmias. An attenuated HRV confers significant prognostic value in end stage renal failure patients (Chandra et al., 2011) as well as in other CRF patients (Oikawa et al., 2009). HRV may also identify patients at increased risk of SCD (Hayano et al., 1999).

Omega-3 PUFA may be beneficial for dialysis patients (Friedman and Moe, 2006) and could also have several beneficial effects in chronic kidney disease patients not treated with dialysis (Fassett et al., 2010). Three studies have examined the effect of omega-3 PUFA on HRV in ESRD patients. The first study included 29 patients and examined the association between the content of omega-3 PUFA in granulocyte membranes and HRV (Christensen et al., 1998). Furthermore, the patients were randomly allocated to dietary supplementation with either 5.2 g of omega-3 PUFA or olive oil for 12 weeks. Only 17 patients completed the study (11 from the omega-3 PUFA group and 6 controls). This hampered comparisons between the two groups, but in the omega-3 PUFA group no increase in HRV was observed after supplementation

whereas the mean RR-interval increased. These patients had very low HRV indices and also low levels of omega-3 PUFA at baseline. This is in accordance with recent data (Madsen et al., 2011). The narrow ranges of both HRV indices and omega-3 PUFA values may partly explain the lack of association between HRV and omega-3 PUFA at baseline. Thus, an increase in the range of omega-3 PUFA levels after dietary supplementation led to a significant positive correlation between omega-3 PUFA in granulocytes and the HRV parameter SDNN. Also, when the patients completing the trial were dichotomized according to their median SDNN, the omega-3 PUFA level was highest among those with the highest SDNN.

In an uncontrolled design, Fiedler et al. (2005) gave 1.2 g of omega-3 PUFA daily for 12 weeks to 11 hemodialysis patients. A number of cardiovascular risk factors were measured and the authors wrote they measured HRV. However, this variability was reported as 60–102 versus 61–105 beats/min before and after supplementation (NS). This was not a continuous recording of the RR-interval, and, therefore, does not provide a measurement of HRV.

In a subgroup of a larger population with ESRD and documented cardiovascular disease, Svensson et al. (2007), randomized 30 patients to 1.7 g of omega-3 PUFA daily or placebo (olive oil) for 3 months. This study could not find any effect on HRV by omega-3 PUFA supplementation but a non-significant trend toward a reduced HR was observed in the omega-3 PUFA arm. It is evident that further studies are warranted regarding the effects of omega-3 PUFA on HRV and autonomic modulation in these high-risk patients.

HEALTHY SUBJECTS

A low HRV seems to predict a poor outcome in healthy populations. In the "Men Born in 1913 Study" randomly selected men aged 50 years had HRV obtained from 10 s ECG strips at entry. During a 10-year follow-up an increased risk of death from IHD during a 10-year was observed in men with a decreased HRV (Tibblin et al., 1975). Also, in the Zutphen study, the 5-year age-adjusted relative risk of mortality for subjects with a low HRV measured from 25 to 30 s ECG strips was 2.1 in middle-aged and 1.4 in elderly men (Dekker et al., 1997), with an inverse association between HRV and the risk of SCD. Data from the Framingham study based on 2-h ECG recordings confirm the predictive value of a decreased HRV in the general population (Tsuji et al., 1994, 1996). Also, Molgaard et al. (1991) found an attenuated HRV in apparently healthy subjects subsequently suffering SCD.

The effect of dietary supplementation with omega-3 PUFA on HRV was examined in healthy subjects (Christensen et al., 1999). In this dose–response study, 60 healthy subjects were randomly divided into three groups receiving either (1) 2.0 g of omega-3 PUFA, (2) 6.6 g of omega-3 PUFA, or (3) placebo (olive oil) daily for 12 weeks. Baseline examination revealed positive correlations between HRV indices and DHA in men, an observation also found by others (Brouwer et al., 2002). Overall, intervention with omega-3 PUFA had no effect on HRV, but if these healthy subjects were dichotomized according to their baseline median SDNN, dietary supplementation with omega-3 PUFA (both 2.0 and 6.6 g) increased RR-interval in those subjects belonging to the lower median. By further stratifying these subjects according to gender, a dose-dependent increase in several HRV indices among men was seen whereas no effect was observed in women. The result from the male participants may emphasize the importance of the actual cellular membrane level of omega-3 PUFA as a major determinant of the risk of SCD (Siscovick et al., 1995; Albert et al., 2002).

Grimsgaard et al. (1998) also showed a reduction in HR (inversely related to RR) after supplementation with DHA to healthy men. Indeed, several studies have shown that omega-3 PUFA reduce HR (Mozaffarian et al., 2005), which may be of importance because an increased HR is strongly associated with a poor cardiovascular outcome (Palatini and Julius, 2004).

In contrast, a study including 84 middle-aged subjects who received 3.5 g of omega-3 PUFA or placebo daily for 12 weeks found no effect of omega-3 PUFA on HRV obtained from short ECG recordings (10 min; Geelen et al., 2003). Compliance was measured in this study. The short recording time excluded the analysis of long-term diurnal variations in heart rhythm which could be of importance. Thus, it is unknown whether the vagal predominance during night time is modifiable by omega-3 PUFA.

A study by Dyerberg et al. (2004) looked at the effect of trans- and omega-3 PUFA on cardiovascular risk markers, among these HRV, in healthy males. The experimental fats were incorporated into bakery products and supplied daily. The omega-3 PUFA group received approximately 4 g of omega-3 PUFA daily. These subjects were healthy well-trained men with a high HRV at baseline and it was not possible to increase their HRV further during 8 weeks of supplementation.

NURSING HOME RESIDENTS

Another study addressing the effect of omega-3 PUFA on HRV included 52 residents from a Mexican nursing home (Holguin et al., 2005). A few of these patients had hypertension or IHD. Omega-3 PUFA 2 g daily or soy oil 2 g daily was supplied. HRV was assessed with 6-min readings obtained every other day for a 2-month run-in period and a 6-month supplementation period. Both supplements improved HRV, with the omega-3 PUFA supplementation being superior to soy oil capsules. There was no analysis of the differences in response between the groups. Nevertheless, this study lends support to the hypothesis that omega-3 PUFA may improve autonomic function.

In a quite similar setting and design as described above the same group included 50 nursing residents from a Mexican nursing home located in an environment where the residents were exposed to particulate matter (Romieu et al., 2005). Romieu et al. reported that omega-3 PUFA supplementation (2 g daily for 6 months) prevented HRV decline related to particulate matter exposure in this study population.

OTHER SUBJECTS

In a study from Norway 53 male (mean age 35 years) inmates from a prison were randomly assigned to intervention and control groups (Hansen et al., 2010). The intervention group received seafood (mainly fatty fish, >8% fat) for dinner three times per week for a period of 6 months. Both groups were requested to eat their usual diet provided by the prison. Blood samples were collected and 5-min ECG recordings were obtained in order to

analyze HRV (HF and LF power) before and after the 6-months study period. Due to various reasons only 13 subjects completed the trial but a significant reduction in the sympathovagal balance (decrease in LF/HF) was observed in the intervention group.

A Korean study evaluated the effects of omega-3 PUFA and simvastatin on lipids and lipoproteins and HRV in patients with mixed dyslipidemia (Kim et al., 2011). Nearly 70% of the patients had hypertension and many received antihypertensive medication. Sixty-two patients were randomized into two treatment groups given a combination treatment with 4 g of omega-3 PUFA and 20 mg of simvastatin daily or a monotherapy of 20 mg simvastatin for 6 weeks. After 6 weeks of intervention an 41% reduction in triglycerides concentration was observed in the omega-3 PUFA groups whereas no significant changes was seen for HRV indices in the time and the frequency domains (24-h recordings). This study had a relatively short intervention period and a longer period of supplementation could be of importance to reach steady state and thereby a possible effect on HRV.

Sudden unexpected death in epilepsy (SUDEP) is a major cause of death in epilepsy. It accounts for up to 20% of mortality and a smaller pilot study examined the effect of omega-3 PUFA supplementation to patients with intractable epilepsy (DeGiorgio et al., 2008). Eleven patients were given 2880 mg omega-3 PUFA daily for 12 weeks and after a 6-week wash-out they were switched to soybean oil (placebo). A non-significant trend toward an increase in time domain HRV measures was observed in only in a few patients with a low baseline. Whether omega-3 PUFA improve HRV in people with epilepsy needs to be further examined in larger controlled clinical trials and it would also be of interest to know if omega-3 PUFA can reduce the high risk of SUDEP in epilepsy.

POPULATION-BASED INVESTIGATIONS, OMEGA-3 PUFA, AND HRV

A large study based on dietary omega-3 PUFA intake and HRV in a well-defined population was published a couple of years ago (Mozaffarian et al., 2008). More than 4000 subjects, aged ≥65 years were included from the Cardiovascular Heart Study. Approximately 25% had DM and 20% had a history of IHD. The time domain HRV indices SDNN and RMSSD were measured from 10-s ECG recordings in 4263 subjects and in a group of 1361 participants, HRV in the time domain and in the frequency domain was derived from 24-h Holter recordings. In a subset of the subjects, plasma phospholipids levels of EPA and DHA was measured and these levels correlated significantly with the fish consumed ($r = 0.55$, $p < 0.001$). According to the fish intake, the participants were divided into five groups and in general, HRV was highest among the participants with the highest fish intake. From the subanalyses of HRV it was suggested that a high fish consumption was associated with an enhanced vagal activity and parasympathetic predominance. Thus, this population-based study seemed to confirm that omega-3 PUFA supplementation can modulate cardiac autonomic function in a favorable way.

Heart rate variability in relation to fruit, vegetable, and fish consumption was reported from the Veterans Administration Normative Aging Study (Park et al., 2009). HRV variables (4-min recordings) were measured among 586 older men with 928 total observations from 2000 to 2007. Dietary intake was evaluated with a self-administered semiquantitative food–frequency questionnaire and categorized into quartiles. No significant association was seen between HRV measures and intakes of other fruit and vegetables, vitamin C, carotenoids, tuna and dark-meat fish, omega-3 PUFA. Thus, these data differed from the Cardiovascular Heart Study despite a similar intake of omega-3 PUFA in both study populations.

Inuit from Nunavik (northern Quebec) consume large amounts of fish and marine mammals. In a cross-sectional study the impact of omega-3 PUFA on resting HR and HRV among Nunavik Inuit adults was assessed also considering mercury exposure and other potential confounders (Valera et al., 2011). HRV data from 2-h Holter recordings was obtained in 181 adults ≥40 years old (109 women and 72 men) living in the 14 coastal villages of Nunavik. Omega-3 PUFA levels were measured in membrane erythrocytes. In women, omega-3 PUFA was significantly associated with HRV indices also after controlling for several potential confounders whereas such associations could not be found in men.

SUMMARY REMARKS

Several studies have found a positive association between omega-3 PUFA and HRV (Christensen et al., 1997, 2001a,b; Brouwer et al., 2002; Mozaffarian et al., 2008; Valera et al., 2011) although data are not consistent (Park et al., 2009). Therefore, interventional studies are of importance and as shown in **Table 2**, 8 of these 20 studies have revealed results supporting a beneficial effect on HRV and in 7 other of these trials a subanalysis of the data pointed at an effect of omega-3 PUFA on HRV. However, 5 of the 20 trials could not confirm an effect on HRV.

The inconsistency of the trial findings could of course be due to a truly absent treatment effect. However, most studies showed some effect and, thus, other considerations should also be taken into account. It is evident that the populations examined are very heterogeneous ranging from infants to nursing home residents. However, despite this heterogeneity, these data still indicate beneficial effects in several trials.

The intervention studies are all with limited sample sizes making conclusions susceptible to statistical errors. Furthermore, the dose of omega-3 PUFA varied considerably as did the length of intervention. Both issues may be of importance because some data suggest a dose-dependent effect of omega-3 PUFA on HRV as well as a certain time may be needed to obtain a steady state of omega-3 PUFA concentration in the relevant tissue.

Of importance are also the different methods of assessing HRV. As seen in **Table 2** HRV data are derived from recordings ranging from 5 min to 24 h. It is obvious that only 24 h recordings measure possible interactions on long-term diurnal variation in HRV. Whether time domain or frequency domain HRV variables are preferable is also an open question but both methods are used and sometimes in combination. Although some evidence (see above) support that the beat-to-beat variation in HR (HRV) reflects corresponding changes in cardiac parasympathetic regulation others have questioned the fact that HRV should reflect parasympathetic regulation (Taylor et al., 2001; Parati et al., 2006; Denver et al., 2007). Therefore, it is of importance to emphasize that HRV does not provide a quantitative measure of cardiac parasympathetic nerve activity (an accurate assessment of nerve activity can only be

obtained from direct nerve recordings) but may provide a very limited qualitative index of changes in cardiac autonomic regulation. Thus, HRV data should always be interpreted with care.

Medications that have an impact on HRV could obscure any effect of omega-3 PUFA on HRV in patients with coronary heart disease or in ESRD patients. Compliance regarding the intake of omega-3 PUFA is also of importance and it is advised that compliance is assured by measuring the incorporation of these fatty acids into phospholipids either in serum or in cell membranes. Another issue is whether it is EPA or DHA or both that might beneficially affect HRV. Most data support DHA as the most important omega-3 PUFA when it comes to modify cardiac autonomic tone. This may be in accordance with the high concentration of DHA in nervous tissue.

Another question of importance is whether it is the HR reducing effect of omega-3 PUFA which is of most importance (Mozaffarian et al., 2005)**?** Such an effect could also be translated into an increased HRV. Recent data (animal study) from Billman and Harris (2011) has suggested that the HRV responses to omega-3 PUFA treatment are more consistent with reductions in the intrinsic pacemaker rate than with alterations in autonomic neural regulation. A previous animal study in rabbits (Verkerk et al., 2009) also found that omega-3 PUFA supplementation reduced pacemaker current and HR. One human study supports a direct effect of omega-3 PUFA on the heart (Harris et al., 2006). Harris et al. found that omega-3 PUFA supplementation reduced HR in cardiac transplant recipients. These hearts were of course denervated and the reduction in HR could thereby not be mediated by a change in vagal tone.

Finally, it can be questioned whether the relatively modest changes in HRV induced by omega-3 PUFA really can explain the improved cardiovascular outcome such as a reduction in SCD. However, in this respect the possible effect on HRV is only one of several beneficial effects of omega-3 PUFA and it may be a combination of these effects which in the end materializes in the improved clinical outcome. In humans you can not isolate the other effects but you can chose to monitor only one of the effects.

CONCLUSION

Both nervous tissue and heart tissue have a high content of omega-3 PUFA (especially DHA) and this may be consistent with the finding that this marine omega-3 fatty acid may modulate cardiac autonomic function as assessed by HRV. Thus, omega-3 PUFA may modulate HRV both at the level of the autonomic nervous system and the heart. Most of the data in this review support that omega-3 PUFA beneficially modulates cardiac autonomic control thereby possibly reducing the risk of arrhythmias. However, data are not consistent perhaps due to a large heterogeneity in the interventional trials such as small sample sizes, different populations, short duration of intake, limited periods of HRV assessment, or variable doses of omega-3 PUFA. Thus, further research is needed to confirm the possible beneficially effect of omega-3 PUFA on HRV. More work is also needed to understand the pathways through which omega-3 PUFA may improve HRV or reduce HR.

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Reduction of heart rate by omega-3 fatty acids and the potential underlying mechanisms

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An elevated resting heart rate is one of the strongest predictors of cardiovascular mortality and is independently associated with sudden cardiac death (SCD). Agents capable of reducing heart rate without significant side effects are therefore of particular interest for the prevention of SCD. Recent human and animal studies have shown that omega-3 fatty acids can reduce heart rate. Our work has shown that omega-3 fatty acids significantly reduce membrane electrical excitability of the cardiac myocyte by lowering its resting membrane potential and the duration of the refractory period through inhibition of ion channels. We propose that these actions may be the underlying mechanisms for the omega-3 fatty acid-induced reduction of heart rate observed in both humans and animals. The heart rate-lowering capability of omega-3 fatty acids may contribute to their preventive effect against SCD.

Keywords: omega-3 fatty acids, cardiac sudden death, heart rate, membrane electrical excitability, ion channel inhibition

INTRODUCTION

The cardioprotective effects of omega-3 fatty acids have become widely recognized. One of the most significant effects is the prevention of sudden cardiac death (SCD) (de Lorgeril et al., 1994; GISSI-Prevenzione, 1999; Marchioli et al., 2002; Leaf et al., 2005, 2003), which is generally defined as death within 1 h of the onset of symptoms, and is most frequently caused by ventricular fibrillation. Although the underlying mechanisms for this preventive effect are not yet well understood, the reduction of heart rate by omega-3 fatty acids may be an important factor contributing to decreased risk for SCD.

An elevated resting heart rate is one of the strongest predictors of cardiovascular mortality. In particular, a resting heart rate of >70–90 beats per minute (bpm) is independently associated with SCD. Multiple prospective studies have shown that even after adjusting for common cardiovascular health-related variables, such as age, weight, smoking, alcohol consumption, diabetes, blood pressure, physical activity, blood cholesterol, medications, and socioeconomic status, elevated heart rate remains a risk factor for SCD and a predictor of time to cardiac death (Shaper et al., 1993; Palatini et al., 1999). In the Framingham cohort, cardiovascular and coronary mortality rates increased with progressively higher resting heart rates irrespective of age or sex, although the fraction of SCD rose sharply in men 35–65 years old (Kannel et al., 1987). In general, similar relationships between heart rate and cardiovascular death exist in men and women, but the association is weaker in women (Kannel et al., 1987; Palatini et al., 1999). Interestingly, heart rate is predictive of SCD in men both with and without a history of ischemic heart disease (IHD) (Wannamethee et al., 1995), and in some cases this relationship is even stronger in men without pre-existing IHD (Shaper et al., 1993).

Given these consistent and robust findings, drugs or supplements that reduce heart rate are of particular interest for preventing SCD. In fact, studies that have examined outcomes for patients with chronic heart failure have revealed that pharmacotherapy to reduce heart rate is associated with better outcomes for at least 5 years (Franke et al., 2012). Beta-adrenergic blockers, cardiotonic agents such as ivabradine, and ACE inhibitors have been prescribed for the purpose of lowering heart rate and reducing mortality, though they may present adverse effects (Arshad et al., 2008; Böhm et al., 2012). Thus, agents that are able to reduce heart rate without significant side effects may be valuable for the prevention of SCD. Omega-3 fatty acids, a class of essential nutrients primarily found in fish oil consisting of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been consistently shown to lower heart rate (Grimsgaard et al., 1998).

The relationship between omega-3 fatty acids and cardiovascular disease is well studied, and has appeared inconsistent at times (Hooper et al., 2006; Kromhout et al., 2010; Rizos et al., 2012). Still, it is important to consider that there is strong mechanistic evidence supporting a protective effect of omega-3 fatty acids on cardiovascular disease. In this review, I will provide an overview of the evidence for the heart rate-lowering effects of omega-3 fatty acids both in animals and humans, and explain how findings from our *in vitro* work provide a likely mechanism by which omega-3 fatty acids act on cardiac myocytes to reduce heart rate.

REDUCTION OF HEART RATE BY OMEGA-3 FATTY ACIDS

The effect of omega-3 fatty acids on heart rate has been observed in many different populations, both with and without cardiovascular disease. A meta-analysis of 30 randomized, double-blind, placebo-controlled trials concluded that fish oil consumption can

significantly reduce heart rate (Mozaffarian et al., 2005). In particular, the effect was greater in people whose baseline heart rate was higher: in the overall pooled estimate, fish oil decreased heart rate by 1.6 bpm compared to placebo, but reduced heart rate by 2.5 bpm in trials with a median baseline heart rate of $\geq$ 69 bpm. Furthermore, the ability of fish oil to reduce heart rate appeared to depend on the length of treatment. When a trial lasted for more than 12 weeks, fish oil reduced heart rate by 2.5 bpm. However, when the trial lasted for less than 12 weeks, fish oil had little effect on heart rate. Interestingly, this meta-analysis also confirmed that heart rate reduction did not vary significantly by fish oil dose (Mozaffarian et al., 2005). Furthermore, another randomized, controlled trial on 18 men with a history of myocardial infarction and ejection fractions of <40% showed that those given omega-3 fatty acids experienced a 5 bpm reduction in resting heart rate and an improved 1-min heart rate recovery after exercise (O'Keefe et al., 2006).

In addition, several large-scale, population-based studies showed that increased dietary fish and omega-3 fatty acid intake was associated with a significant reduction in heart rate. Dallongeville et al. (2003) analyzed 2 years of data on 9758 men without coronary heart disease from France and Ireland, grouping the men into four statistical categories based on how much fish they consumed per week (less than once, once, twice, and more than twice/week). They found that heart rate decreased across the categories of fish intake and was lower in fish consumers than in non-consumers, even after adjustments for age, location, level of education, physical activity, smoking habits, alcohol consumption, body mass index, and antiarrhythmic medications (Dallongeville et al., 2003). Studies by Mozaffarian and colleagues further examined the associations between fish intake and a variety of cardiac measures (Mozaffarian et al., 2006a,b). Their results showed that high fish consumption is associated with a heart rate reduction of approximately 3.2 bpm. They also found that an estimated 1 g/day higher EPA + DHA intake was associated with a heart rate reduction of 2.3 bpm. Functionally, this improvement in heart rate (−3.2 bpm) corresponds to a ∼7.5% lower risk of SCD (Mozaffarian et al., 2006b).

Fish oil also effectively reduces heart rate during times of increased cardiac demand such as exercise. A study of 25 Australian football players revealed that 6g/day of fish oil reduced heart rate during submaximal exercise over a period of 5 weeks (Buckley et al., 2009). Likewise, another randomized, placebo-controlled study of 16 exceptionally fit male cyclists taking 8g/day of fish oil for 8 weeks also found a reduction in heart rate during exercise. Heart rate during incremental workloads to exhaustion was lowered, as was peak heart rate, oxygen consumption, and heart rate during steady submaximal exercise (Peoples et al., 2008). However, decreased heart rate from fish oil during exercise is not contingent on physical fitness; in a study of 65 sedentary, overweight volunteers who consumed tuna fish oil for 12 weeks, resting heart rate and heart rate response to submaximal exercise were decreased (Ninio et al., 2008). Thus, fish oil reduced heart rate both at rest and during the stress of exercise, irrespective of the relative fitness level of the participant.

Another set of interesting findings comes from a population perhaps the least likely to experience cardiovascular illness: infants. Term infants treated with varying amounts of DHA in their formulas for 12 months show that DHA supplementation reduces heart rate compared to infants whose formula does not contain DHA, with no evidence of a dose response (Pivik et al., 2009; Colombo et al., 2011). These data are noteworthy in that they reinforce the non-specific impact of fish oil on heart rate, and suggest that almost any cohort may benefit from fish oil in this manner.

Finally, Harris and colleagues performed a small prospective study that provides a valuable indication of which mechanisms are likely to underlie the omega-3 fatty acid-driven reduction in heart rate. The group enrolled heart transplant patients, ensuring that their transplants had occurred more than three months prior and there had been no transplant-related hospitalizations (Harris et al., 2006). The revealing aspect of this study is that transplanted hearts are functionally denervated of the vagal nerve, and thus devoid of sympathetic and parasympathetic inputs. The patients were randomly assigned to receive either a corn oil placebo or EPA/DHA for 4–6 months, and at the end of the study the patients in the omega-3 fatty acid group had heart rates on average 5.4 bpm lower than baseline, whereas the corn oil group showed no change (Harris et al., 2006). These findings suggest that omega-3 fatty acids impact heart rate at the level of the myocardium itself, and are in particular consistent with the idea that the voltage-gated ion channels that control the pacemaker currents in the heart are influenced by omega-3 fatty acids.

Similar reductions in heart rate due to omega-3 fatty acids have been observed in animals. In a rat model, animals fed a DHA-enriched diet had lower heart rates than animals fed a control diet, a pattern that was achieved by 2 months and maintained until the end of the 32-week study (Ayalew-Pervanchon et al., 2007). In a hyperinsulinemic model, rats fed a diet containing DHA showed lower heart rates and a shortened QT interval as compared to rats fed and EPA-rich diet (Rousseau et al., 2003). Similarly, in rabbits fed a diet enriched with 2.5% (w/w) fish oil for three weeks, sinus cycle length and heart rate were reduced compared to animals fed a 2.5% oleic sunflower oil for the same period of time (Verkerk et al., 2009). A series of studies by Billman and colleagues has also provided important information about omega-3 fatty acids and heart function. Omega-3 fatty acid infusions into 13 intact, conscious, exercising dogs highly susceptible to ischemia-induced ventricular fibrillation were able to prevent ventricular fibrillation in 10 of the 13 dogs tested. The antiarrhythmic effect was associated with slowing of the heart rate, shortening of the QT interval, reduction of the left ventricular systolic pressure, and prolongation of the electrocardiographic atrial-ventricular conduction time (PR interval) (Billman et al., 1997). Additional work showed that dietary omega-3 supplementation reduces resting heart rate and increases heart rate variability in dogs with previous but healed myocardial infarction, as well as in dogs that are either susceptible or resistant to ventricular fibrillation (Billman and Harris, 2011; Billman, 2012). Moreover, these reductions in baseline heart rate were maintained during challenges (i.e., exercise or acute myocardial ischemia), but omega-3 did not alter the amount of change induced by challenge; these findings are consistent with the idea that omega-3 supplementation

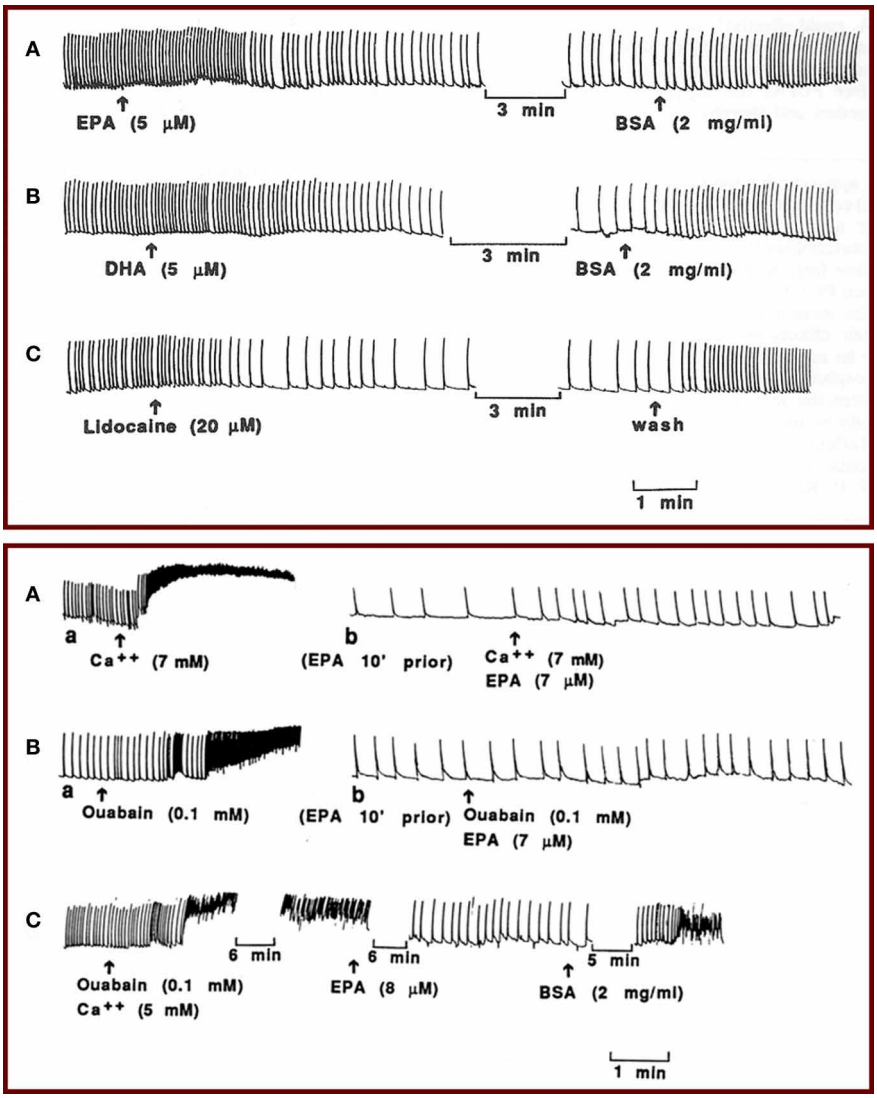

FIGURE 1 | (Top panel) Effects of EPA and DHA on the contraction of isolated neonatal rat cardiomyocytes. Perfusion of the myocytes with 5 μM EPA **(A)** or 5 μM DHA **(B)** reduced the beating rate by 50% within 2 min, and addition of BSA to the perfusion solution quickly reversed the effect. Tracing **(C)** shows a similar effect of lidocaine (20 μM) on the contraction of the cardiac myocytes. **(Bottom panel)** Tracings show prevention and termination of arrhythmia by EPA. Perfusion of the myocytes with a solution containing 7 AM Ca^{2+} (**A**, a) or 0.1 mM ouabain (**B**, a) induced contracture and fibrillation before perfusion with EPA. Washing the cells with medium (Ca^{2+} = 1.2 mM) returned the fibrillations to the original beating rate (not shown). Then the cells were perfused with medium containing 7 μM EPA. After 5–8 min, when the beating rate was slowed, addition of 7 mM Ca^{2+} (**A**, b) or 0.1 mM ouabain (**B**, b) failed to induce contracture or fibrillation in the same cells. The slow beating rates were subsequently returned to the original rates by perfusion with BSA (not shown). **(C)** Alternatively, after induction of fibrillation by ouabain (0.1 mM) plus Ca^{2+} (5 mM), addition of IEPA (8 μM) terminated the fibrillation and led to slow beating, and subsequent addition of BSA (2 mg/ml), still in the presence of ouabain and high external Ca^{2+} concentration, reinstated fibrillation.

impacts intrinsic heart rate rather than autonomic regulation of the heart. Interestingly, there is also evidence that under certain conditions omega-3 may actually be pro-arrhythmic by reducing myocyte excitability during acute regional ischemia (Coronel et al., 2007).

Overall, it is apparent that a wide range of human and animal subjects, with or without cardiac disease, all respond to omega-3 fatty acid supplementation with reductions in resting and stress-induced heart rates. These findings suggest a highly consistent and robust effect of omega-3 fatty acids on heart rate.

POTENTIAL MECHANISMS UNDERLYING THE HEART RATE-LOWERING EFFECT OF OMEGA-3 FATTY ACIDS

Although omega-3 fatty acids may reduce heart rate through several different mechanisms, our early studies demonstrated a direct effect of omega-3 fatty acids on cardiac cell membrane electrical excitability that contributes to reduced heart rate. We used isolated, neonatal rat cardiac myocytes that retain spontaneous beating behavior, allowing us to assess the effect of EPA and DHA on contraction as well as electrophysiological activity without neural or hormonal input. We found that EPA and DHA

promptly reduced the contraction rate of the cardiac myocytes by 50–80% without a significant change in the amplitude of the contractions. This effect of omega-3 fatty acids on the excitability of the cells was similar to that produced by the class I antiarrhythmic drug lidocaine (**Figure 1**, top) (Kang and Leaf, 1994). In addition, we showed that EPA and DHA can prevent as well as terminate fibrillation, characterized by chaotic, asynchronous beating and contractures, induced either by high extracellular calcium concentrations and/or ouabain (**Figure 1**, bottom) (Kang and Leaf, 1994). Inhibitors of fatty acid metabolism, however, have no effect on omega-3 fatty acid-induced heart cell contraction, indicating that the free fatty acids do not need to be metabolized into byproducts to cause a reduction in heart rate (Kang and Leaf, 1994). The omega-3 fatty acid-induced reduction in the beating rate could be readily reversed by cell perfusion with fatty acid-free bovine serum albumin, indicating that omega-3 fatty acids likely do not need to be incorporated into the membrane phospholipid or covalently linked to membrane components in order to be effective. These results suggest that omega-3 fatty acids in their free form can suppress the automaticity of cardiac contraction and thereby exert their heart rate-lowering effects.

The reduction of electrical excitability of cardiac myocytes by omega-3 fatty acids can be demonstrated directly by their response to electrical pacing (Kang and Leaf, 1996). As shown in **Figure 2**, prior to addition of EPA to the cells, application of a series of stimulating impulses elicited a rapid beating synchronized with the impulse rate. 3–5 min after perfusion of the cells with 15 uM EPA, when a slowing of the beating rate had occurred, application of the same (15 V) or even stronger electrical stimuli failed to boost the beating rate. When the cells were washed with medium containing delipidated BSA (2 mg/ml) for 2–3 min, stimulation of the cells with 15 V field strength induced a response similar to that observed prior to addition of EPA (**Figure 2**) (Kang and Leaf, 1996). These results further suggest that omega-3 fatty acids have an inhibitory effect on the electrical automaticity/excitability of the cardiac myocytes.

To better elucidate the mechanism of action of omega-3 fatty acids on heart rate, we employed a patch-clamp technique to examine the electrophysiological activity in isolated neonatal rat cardiac myocytes. First, we induced the action potential in the myocytes exposed to EPA or DHA and measured the strength of the current required to elicit an action potential (Kang et al., 1995). We found that EPA increased the strength of the depolarizing current needed to provoke an action potential and lengthened the cycle of excitability. These changes were due to an increase in the threshold for action potential and a more negative resting membrane potential. There was a progressive prolongation of intervals between spontaneous action potentials and a slowed rate of phase 4 depolarization (**Figure 3**) (Kang et al., 1995). These results demonstrate that omega-3 fatty acids can indeed reduce membrane electrical excitability and provide an electrophysiological basis for the heart rate-lowering effects of free omega-3 fatty acids. These findings are consistent with the observations that omega-3 fatty acids can profoundly reduce the contraction rate of cardiac myocytes (Kang and Leaf, 1994).

At this point in our research, the manner by which omega-3 fatty acids reduce membrane excitability was still unclear. Therefore, we tested the effects of omega-3 fatty acids on single ion channel activity in neonatal rat cardiac myocytes. The results demonstrated a prompt inhibitory action of omega-3 fatty acids on the Na+ currents through fast sodium channels responsible for the phase 0 of the action potential in isolated neonatal rat

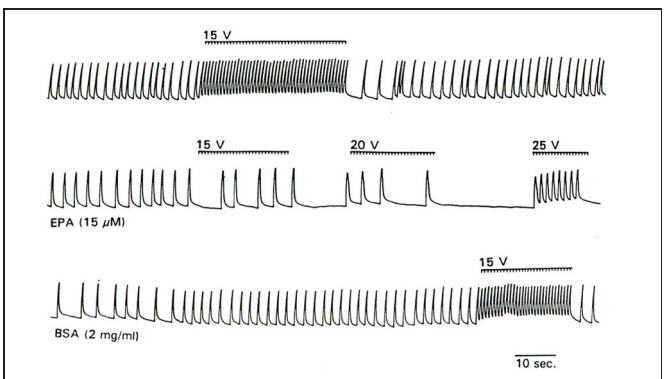

FIGURE 2 | Effect of EPA on the response of cultured myocytes to electrical pacing. Trace shows the response of cell contraction to the electrical stimuli (as indicated by the bars above the contraction trace) before perfusion with EPA (top trace), during perfusion with 15 μM EPA (middle trace) and after washout with BSA.

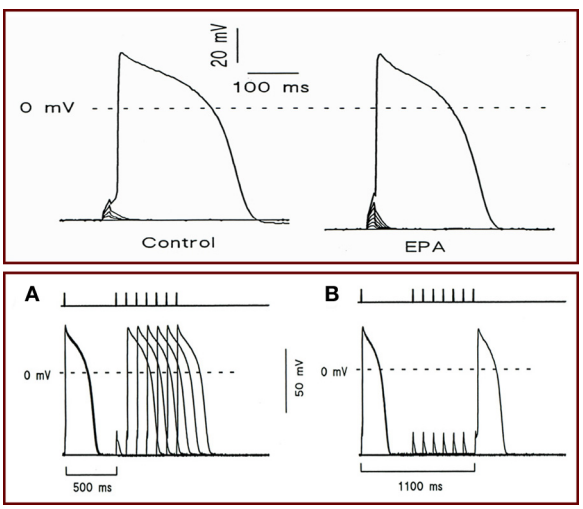

FIGURE 3 | (Top panel) Effect of EPA on holding voltage at constant imposed current, threshold voltage, and current required to attain action-potential threshold. Holding potential was initially set at −70 mV, and cells were stimulated with a series of depolarization pulses in 3- or 4-pA increment at 10 s intervals. Recordings showing the holding transmembrane potentials, the threshold potentials, and the currents required to elicit an action potential in a cell before (Left) and after (Right) exposure to 10 μM EPA. **(Bottom panel)** The effect of EPA on the cycle length of excitability. Cells were given a pair of superthreshold electrical stimuli at 0.1 Hz (necessary to elicit action potential in the presence of EPA) with various intervals from 500 to 1500 ms in 100 ms increments. Bars at top indicate the time intervals between two stimuli. **(A)** Recording showing the cycle length (600 ms) before 10 μM EPA exposure. **(B)** Recording showing cycle length (1100 ms) 3 min after EPA exposure.

cardiac myocytes. The inhibition of this ion channel was dose, time, and voltage dependent, but not use dependent (**Figure 4**) (Xiao et al., 1995). Subsequent studies have demonstrated that other ion channels, such as calcium channels, can also be affected by omega-3 fatty acids to varying degrees (Leaf, 2001). These findings provided the ionic basis for the marked electrophysiological effects of omega-3 fatty acids on myocytes, and explained why omega-3 fatty acids are capable of reducing heart rate.

CONCLUSIONS

Regulation of heart rate in humans is highly complex. Sympathetic output, vagal tone, and systolic and diastolic left ventricular function are only a few of the factors that contribute to the regulation of heart rate. While omega-3 fatty acids could potentially affect any or all of these factors, our studies strongly suggest a direct impact of omega-3 fatty acids (Leaf et al., 1999a,b). Our cellular work has shown that omega-3 fatty acids significantly reduce membrane electrical excitability of the cardiac myocyte by lowering its resting membrane potential and the duration of the refractory period through inhibition of ion channels. We propose that these actions may be the underlying mechanisms for the omega-3 fatty acid-induced reduction of heart rate observed in both humans and animals. Given the close relationship between heart rate and SCD, the direct impact of omega-3 fatty acids on the cardiac membrane to reduce heart rate may be a critical determinant of the preventive effect of omega-3 fatty acids against SCD. Thus, increasing intake of omega-3 fatty acids would likely benefit individuals at risk for SCD.

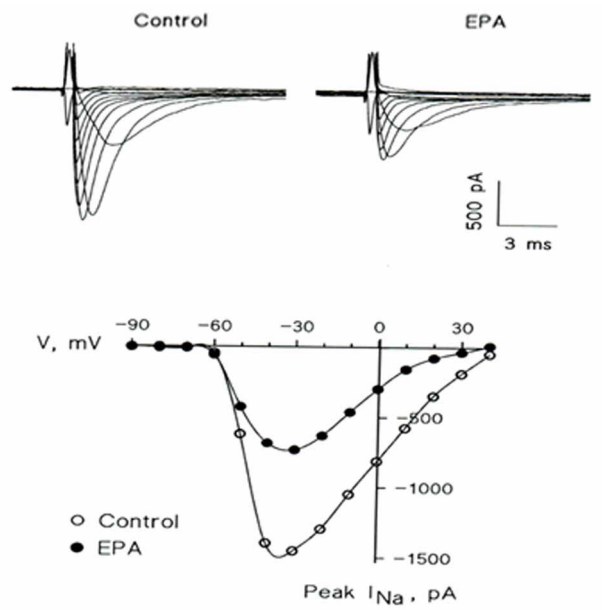

FIGURE 4 | EPA suppresses the voltage-activated Na+ current (I_{Na}) in a representative cultured neonatal rat ventricular myocyte. (Upper) Superimposed traces in the absence (Control) and presence (EPA) of 5 μM EPA. The currents were elicited by 10 mV increment voltage steps (20 ms; 0.2 Hz) from a holding potential of −80 mV down to −90 mV and up to 40 mV. (Lower) Current-voltage relations of peak I_{Na} in the absence (○) and presence (●) of 5 μM EPA.

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Effect of dietary omega-3 polyunsaturated fatty acids on heart rate and heart rate variability in animals susceptible or resistant to ventricular fibrillation

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The consumption of omega-3 polyunsaturated fatty acids (n−3 PUFAs) has been reported to reduce cardiac mortality following myocardial infarction as well as to decrease resting heart rate (HR) and increase HR variability (HRV). However, it has not been established whether *n*−3 PUFAs exhibit the same actions on HR and HRV in individuals known to be either susceptible or resistant to ventricular fibrillation (VF). Therefore, HR and HRV (high frequency and total R–R interval variability) were evaluated before and 3 months after *n*−3 PUFA treatment in dogs with healed myocardial infarction that were either susceptible (VF+, $n = 31$) or resistant (VF−, $n = 31$) to ventricular tachyarrhythmias induced by a 2-min coronary artery occlusion during the last minute of a submaximal exercise test. HR and HRV were evaluated at rest, during submaximal exercise and in response to acute myocardial ischemia at rest before and after either placebo (1 g/day, corn oil, VF+, $n = 9$; VF− $n = 8$) or *n*−3 PUFA (docosahexaenoic acid + eicosapentaenoic acid ethyl esters, 1–4 g/day, VF+, $n = 22$; VF−, $n = 23$) treatment for 3 months. The *n*−3 PUFA treatment elicited similar increases in red blood cell membrane, right atrial, and left ventricular *n*−3 PUFA levels in both the VF+ and VF− dogs. The *n*−3 PUFA treatment also provoked similar reductions in baseline HR and increases in baseline HRV in both groups that resulted in parallel shifts in the response to either exercise or acute myocardial ischemia (that is, the change in these variables induced by physiological challenges was not altered after *n*−3 PUFA treatment). These data demonstrate that dietary *n*−3 PUFA decreased HR and increased HRV to a similar extent in animals known to be prone to or resistant to malignant cardiac tachyarrhythmias.

Keywords: parasympathetic nervous system, exercise, myocardial ischemia, fish oil, docosahexaenoic acid, eicosapentaenoic acid

INTRODUCTION

There is strong association between abnormal cardiac autonomic regulation and an increased risk for sudden cardiac death (Billman, 2009). In particular, both patients and animals that exhibit large reductions in cardiac parasympathetic activity coupled with an enhanced sympathetic activation following myocardial infarction have an increased incidence of malignant ventricular tachyarrhythmias and sudden cardiac death (Billman, 2009). Therefore, therapeutic interventions that improve cardiac balance could protect against sudden death in high-risk patient populations.

A number of experimental and clinical studies report that dietary omega-3 polyunsaturated fatty acids (*n*−3 PUFAs; Christensen et al., 1999; Christensen and Schmidt, 2007; Christensen, 2011) or the acute intravenous administration (Billman et al., 1994) of these lipids can both lower resting heart rate (HR) and increase resting HR variability (HRV), data consistent with an enhanced baseline cardiac parasympathetic tone (Billman, 2009, 2011). It has been proposed that the cardiovascular benefits ascribed to dietary *n*−3 PUFAs could result, at least in part, from these reductions in HR and the corresponding putative improvements in cardiac autonomic balance (Christensen et al., 1999; Christensen and Schmidt, 2007; Christensen, 2011) or intrinsic rate (Laustiola et al., 1986; Kang and Leaf, 1994; Harris et al., 2006; Verkerk et al., 2009; Billman and Harris, 2011). Billman and Harris (2011) recently demonstrated that *n*−3 PUFA supplements decrease baseline HR and increased baseline HRV in animals with healed myocardial infarctions but did not alter the response to physiological challenges (exercise or acute myocardial ischemia). However, they did not determine whether the *n*−3 PUFA treatment elicited different actions in animals that were known to be either resistant or susceptible to ventricular fibrillation (VF). It is possible that those individuals with the greatest impairment in cardiac autonomic regulation (and at the greatest risk for adverse cardiac events) may exhibit larger changes in HRV following *n*−3 PUFA treatment than patients with well-preserved autonomic function.

It was, therefore, the purpose of the present study to evaluate the effects of dietary *n*−3 PUFAs (1–4 g/day for 3 months) on the HR and the HRV responses to physiological stressors (exercise or acute myocardial ischemia) in dogs with healed myocardial

infarctions that had been identified as either being susceptible (VF+) or resistant (VF−) to the induction of malignant ventricular tachyarrhythmias. In particular, the hypothesis that dietary *n*−3 PUFA supplements would produce different HR and HRV responses to physiologic challenges in VF+ and VF− dogs was tested.

MATERIALS AND METHODS

All the animal procedures were approved by the Ohio State University Institutional Animal Care and Use Committee and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Archived data from 62 heartworm free mixed breed dogs (2- to 3-year-old, male $n = 20$, female $n = 42$) weighing 19.9 ± 0.4 kg (range 12.5–25.8 kg) that were part of an ongoing investigation of the cardiovascular effects of dietary *n*−3 PUFA (Billman et al., 2010, 2012; Billman and Harris, 2011) were used in the present study. The sole selection criterion was an ECG signal of sufficient quality to determine HRV both at baseline and in response to physiological challenges (i.e., exercise or acute myocardial ischemia).

SURGICAL PREPARATION

The animals were anesthetized and instrumented to measure a ventricular electrogram (from which HR and HRV were subsequently determined) and left circumflex coronary artery blood flow as previously described (Billman et al., 1982; Schwartz et al., 1984; Billman, 2006). A hydraulic vascular occluder (Model OC3, *In vivo* Metric, Healdsburg, CA, USA) was placed around the left circumflex coronary artery and used to induce acute myocardial ischemia for the coronary occlusion experiments described below (see the HRV protocols). 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 (Billman, 2006]. This vessel was partially occluded for 20 min and then tied off. The dogs were given analgesic, antibiotic, and anti-arrhythmic therapy to alleviate post-operative pain, to prevent post-operative infection, and to reduce acute arrhythmias associated with the myocardial infarction as has been previously described (Billman et al., 1982; Schwartz et al., 1984; Billman, 2006).

EXERCISE PLUS ISCHEMIA TEST

The studies began 3–4 weeks after the production of the myocardial infarction. The susceptibility to VF was tested as previously described (Billman et al., 1982; Schwartz et al., 1984; Billman, 2006). Briefly, the animals ran on a motor-driven treadmill while workload progressively increased until a HR 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 (Adult Stat-padz, Zoll Medical, Burlington, MA, USA) were placed across the animal's chest so that electrical defibrillation (Zoll M series defibrillator) 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. In the present study, 31 dogs developed VF (susceptible, VF+) while 31 did not (resistant, VF−).

HEART RATE VARIABILITY PROTOCOLS

Heart rate variability was calculated using a Delta-Biometrics vagal tone monitor triggering off the electrocardiogram R–R interval (Urbana-Champaign, IL, USA). This device employs the time-series signal processing techniques as developed by Porges to estimate the amplitude of respiratory sinus arrhythmia [the high frequency, HF component of R–R interval variability (Porges, 1986)]. Details of this analysis have been described previously (Billman and Hoskins, 1989; Billman and Dujardin, 1990; Houle and Billman, 1999). Data were averaged over 30 s intervals either during exercise or the coronary occlusion. The following indices of HRV were determined: Vagal Tone Index, the HF (0.24–1.04 Hz) component of R–R interval variability, and the SD of the R–R intervals (a marker of total variability) for the same 30 s time periods.

First, over the period of 3–5 days, the dogs learned to run on a motor-driven treadmill. The cardiac response to submaximal (i.e., 60–70% of maximal HR) exercise was then evaluated as follows: exercise lasted a total of 18 min with workload increasing every 3-min. The protocol began with a 3-min "warm-up" period, during which the dogs ran at 4.8 kph at 0% grade. The speed was then increased to 6.4 kph, and the grade increased every 3-min (0, 4, 8, 12, and 16%). The submaximal exercise test was repeated three times (one/day). On a subsequent day, with the dogs lying quietly unrestrained on a table, a 2-min left circumflex coronary occlusion was made. Left circumflex blood flow, HR, and HRV were monitored continuously throughout the exercise or occlusion studies. The submaximal exercise and the coronary occlusion at rest studies were performed both before and after 3 months of treatment with either placebo (1 g/day corn oil) or daily *n*−3 PUFA capsules (1–4 g/day).

DIETARY OMEGA-3 POLYUNSATURATED FATTY ACID PROTOCOL

The dogs were placed on a diet that did not contain any *n*−3 PUFAs (Harlan Teklad, Harlan Laboratories, Inc., Indianapolis, IN, USA) beginning 1 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 ($n = 17$: S, $n = 9$; R, $n = 8$); *n*−3 PUFA (1–4 g/day, $n = 45$: S, $n = 22$; R, $n = 23$). The dogs were given supplements similar to those used in the GISSI-Prevenzione study (Di Stasi et al., 2004). 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, USA). 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 a.m. each day, 7 days per week for 3 months).

RED BLOOD CELL AND CARDIAC TISSUE FATTY ACID ANALYSIS

Fasting blood samples (5 ml) were drawn into EDTA tubes from a cephalic vein between 8:00 and 9:00 a.m. 1 day prior to the initiation of the treatment (placebo or *n*−3 PUFA) and when tissue was harvested at the end of the study (∼14 weeks after the treatment began). Right atrial and left ventricular tissue were obtained when the hearts were harvested; the tissue and red blood cells (RBC) were flash frozen in liquid nitrogen; and stored at −80°C for future analysis.

Red blood cell and phospholipids from cardiac tissue were analyzed for fatty acid composition using previously described techniques (Bligh and Dyer, 1959; Morrison and Smith, 1964). The samples were analyzed by gas chromatography using a GC2010-FID (Shimadzu Corporation, Columbia, MD, USA) equipped with a 100-mm capillary column (SP-2560, Supelco, Bellefonte, PA, USA). Fatty acids of interest were identified by comparison with known standards and expressed as a percent of total fatty acids. The coefficient of variation for the RBC EPA + DHA assays was <5%.

DATA ANALYSIS

All data are reported as mean ± SEM. The data were digitized (1 kHz) and recorded using a Biopac MP-100 data acquisition system (Biopac Systems, Inc., Goleta, CA, USA). The HR and HRV data were averaged over 30 s intervals either during exercise or the coronary occlusion. ECG variables were averaged over the last five beats before and 60 s after the onset of the coronary occlusion. QT interval was corrected for changes in HR using Van de Water's correction formula [$QTc = QT - 87(60/HR - 1)$] (Van de Water et al., 1989).

The data were compared using ANOVA for repeated measures (NCSS statistical software, Kaysville, UT, USA). For example, the effects of *n*−3 PUFAs on the HR and HRV response to either submaximal exercise or the coronary artery occlusion at rest for the resistant or susceptible dogs were analyzed using a three factor ANOVA [pre–post (two levels) × dose (placebo vs. *n*−3 PUFA) × time (exercise seven levels or occlusion six levels) with repeated measures on two factors (pre–post and time)]. The effect of *n*−3 PUFA on ECG variables before and 60 s after coronary occlusion for the resistant or susceptible dogs were evaluated using a three factor [pre–post (two levels), dose (two levels), and occlusion time (two levels, before and 60 s after occlusion onset points)] ANOVA with repeated measure on two (pre–post and occlusion time) factors. Homogeneity of covariance (sphericity assumption, equal correlates between the treatments) was tested using Mauchly's test and, if appropriate, adjusted using Huynh–Feldt correction. RBC and cardiac tissue lipid compositions were compared using a three factor ANOVA [group (VF+ vs. VF−), dose (placebo vs. *n*−3 PUFA), pre–post] with repeated measures on one factor (pre–post) or a two factor ANOVA [group (susceptible vs. resistant), dose (placebo vs. *n*−3 PUFA], respectively. If the F value exceeded a critical value ($P < 0.05$), *post hoc* comparisons of the data were then made using Tukey–Kramer Multiple-Comparison Test.

RESULTS

EFFECT OF *n*−3 PUFA ON RED BLOOD CELL AND CARDIAC TISSUE FATTY ACID CONTENT

In agreement with previous studies (Billman et al., 2010; Billman, 2011), *n*−3 PUFA supplements elicited significant (dose effect, pre–post, and dose × pre–post interaction, all $P < 10^{-6}$) increases in RBC membrane EPA, DHA, and the omega-3 index (EPA + DHA) as compared to the placebo treated animals (**Table 1**). Similar increases in EPA, DHA, and the omega-3 index were noted for both the susceptible and the resistant dogs (i.e., there were no significant group, group × dose interactions, or group × pre–post interactions). Thus, *n*−3 PUFA treatment increased RBC *n*−3 PUFA content to a similar extent in both the VF+ and VF− dogs while lipid composition did not change during the 3-month study period in the placebo treated dogs in either group. In a similar manner, right atrial and left ventricular *n*−3 PUFA content was significantly higher ($P < 10^{-6}$) in *n*−3 PUFA compared to the placebo treated animals (**Table 2**). Once again these increases were similar in both the VF+ and VF− groups (i.e., there were no group or group × dose interactions for the either the LV or for the RA tissue).

EFFECT OF *n*−3 PUFA ON BASELINE HEART RATE AND HEART RATE VARIABILITY

The effect of *n*−3 PUFA or placebo on baseline (i.e., before a physiological challenge) HR and HRV are listed in **Table 3**. The *n*−3 PUFA treatment elicited significant reductions in HR ($P < 0.0002$)

Table 1 | Red blood cell omega-3 polyunsaturated fatty acid content.

	EPA		DHA		Omega-3 index	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
PLACEBO						
VF− (*n* = 4)	0.17 ± 0.01	0.15 ± 0.03	0.30 ± 0.05	0.13 ± 0.02	0.47 ± 0.04	0.28 ± 0.05
VF+ (*n* = 7)	0.14 ± 0.01	0.20 ± 0.02	0.20 ± 0.01	0.24 ± 0.05	0.34 ± 0.02	0.41 ± 0.04
***n*−3 PUFA**						
VF− (*n* = 23)	0.18 ± 0.02	3.28 ± 0.29*+	0.23 ± 0.02	2.63 ± 0.16*+	0.40 ± 0.04	5.94 ± 0.42*+
VF+ (*n* = 22)	0.20 ± 0.01	2.79 ± 0.33*+	0.27 ± 0.05	2.52 ± 0.19*+	0.52 ± 0.07	5.31 ± 0.45*+

*All values are expressed of % of the total lipid content. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; omega-3 index, EPA + DHA, *P < 0.01 pre vs. post, +P < 0.01 placebo vs. n−3 PUFA (omega-3 polyunsaturated fatty acids). There were no significant differences between VF− (resistant to ventricular fibrillation) and VF+ (susceptible to ventricular fibrillation) for either the placebo or n−3 PUFA treated animals.*

Table 2 | Cardiac tissue omega-3 polyunsaturated fatty acid content.

	EPA	DHA	Omega-3 index
RIGHT ATRIUM			
Placebo			
VF−	0.11 ± 0.08	0.16 ± 0.10	0.27 ± 0.18 (*n* = 3)
VF+	0.36 ± 0.12	0.58 ± 0.17	0.91 ± 0.25 (*n* = 6)
n−3 PUFA			
VF−	2.04 ± 0.31*	2.89 ± 0.33*	4.92 ± 0.59* (*n* = 23)
VF+	1.62 ± 0.25*	2.80 ± 0.37*	4.41 ± 0.61* (*n* = 22)
LEFT VENTRICLE			
Placebo			
VF−	0.12 ± 0.06	0.19 ± 0.06	0.31 ± 0.11 (*n* = 3)
VF+	0.34 ± 0.05	0.45 ± 0.10	0.80 ± 0.13 (*n* = 6)
n−3 PUFA			
VF−	2.98 ± 0.30*	3.00 ± 0.17*	5.98 ± 0.43* (*n* = 23)
VF+	2.27 ± 0.28*	2.80 ± 0.20*	4.41 ± 0.43* (*n* = 21)

*All values are expressed of % of the total lipid content. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; omega-3 index, EPA + DHA, *P < 0.01 placebo vs. n−3 PUFA (omega-3 polyunsaturated fatty acids). There were no significant differences between VF− (resistant to ventricular fibrillation) and VF+ (susceptible to ventricular fibrillation) for either the placebo or n−3 PUFA treated animals.*

Table 3 | Effect of dietary omega-3 polyunsaturated fatty acids on heart rate and heart rate variability.

	Pre-treatment	Post-treatment
HEART RATE (BEATS/MIN)		
VF+		
Placebo (*n* = 9)	122.4 ± 4.7	121.6 ± 4.20
n−3 PUFA (*n* = 22)	123.3 ± 4.5	112.6 ± 4.2*
VF−		
Placebo (*n* = 8)	123.3 ± 5.2	127.0 ± 6.0
n−3 PUFA (*n* = 23)	122.3 ± 4.1	106.9 ± 3.8*
HIGH FREQUENCY (0.24–1.04 Hz) VARIABILITY (ln ms^2)		
VF+		
Placebo	6.7 ± 0.3	6.3 ± 0.5
n−3 PUFA	7.0 ± 0.3	7.5 ± 0.2*
VF−		
Placebo	7.0 ± 0.3	6.7 ± 0.4
n−3 PUFA	6.7 ± 0.3	7.7 ± 0.3*
R–R INTERVAL SD (ms)		
VF+		
Placebo	60.2 ± 6.9	67.8 ± 10.2
n−3 PUFA	59.0 ± 6.6	69.8 ± 5.8
VF−		
Placebo	69.6 ± 8.2	68.8 ± 9.4
n−3 PUFA	65.9 ± 10.3	74.8 ± 6.3

**P < 0.01 Pre-treatment vs. Post-treatment; n−3 PUFA, omega-3 polyunsaturated fatty acids; VF+, susceptible to ventricular fibrillation; VF−, resistant to ventricular fibrillation.*

that were accompanied by a corresponding increase in the HF component (HF, 0.24–1.04 Hz) of R–R interval variability ($P < 0.002$) in both the VF− and VF+ animals. However, total beat-to-beat variability as measured by the SD of R–R variability was not altered by *n*−3 PUFA treatment. There were no differences noted between the VF+ and VF− dogs (no significant group effect or group × pre–post interactions). In contrast, placebo treatment did not alter either baseline HR, HF, or SD in either the VF− or in the VF+ groups (**Table 3**). Thus, *n*−3 PUFA treatment provoked changes in resting HR and HRV in both VF− and VF+ dogs that were consistent with either an enhanced cardiac vagal regulation (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Berntson et al., 1997; Billman, 2009, 2011) or a change in baseline intrinsic rate (Verkerk et al., 2009; Billman and Harris, 2011).

EFFECT OF *n*−3 PUFA ON THE ECG VARIABLES, HEART RATE, AND HEART RATE VARIABILITY RESPONSE TO ACUTE MYOCARDIAL ISCHEMIA

The effects of the placebo and the *n*−3 PUFA treatment on ECG parameters at baseline and in response to coronary artery occlusion for the VF+ and VF− dogs are listed in **Table 4**. The coronary occlusion provoked significant increases in HR (both VF+ and VF−, $P < 10^{-6}$) and the descending portion of the T wave (VF+ only, $P < 0.00003$), a marker of the dispersion of repolarization (Yan and Antzelevitch, 1998; Opthof et al., 2007) while eliciting decreases in PR interval (VF+, $P < 0.0001$; VF−, $P < 0.002$). No other ECG variable was affected by the ischemia. The *n*−3 PUFA treatment (but not placebo) elicited significant reductions in HR (VF+, $P < 0.04$; VF−, $P < 0.04$) and increases in PR interval (VF+, $P < 0.02$; VF−, $P < 0.03$), changes that were maintained during the coronary occlusion. Interestingly, pre-occlusion QTc interval increased in both the placebo and *n*−3 PUFA treatment (VF+, $P < 0.02$; VF−, $P < 0.0001$) at the end of the 3-month treatment period as compared to values obtained before the treatment began.

The effects of placebo and the *n*−3 PUFA treatment on the HR and HRV (HF only) response to the acute myocardial ischemia are displayed for VF+ and VF− dogs in **Figures 1** and **2**, respectively. In agreement with previous studies (Collins and Billman, 1989; Halliwill et al., 1998; Houle and Billman, 1999; Billman, 2006; Billman and Kukielka, 2006), the coronary occlusion significantly increased HR (occlusion time effect, $P < 10^{-6}$) and decreased HRV (both HF and SD, occlusion time effect, $P < 10^{-6}$) in the placebo and in the *n*−3 PUFA treated animals. HR was significantly lower (pre–post, VF− $P < 0.0004$; VF+ $P < 0.02$), and both HF (pre–post, VF− $P < 0.0001$; VF+ $P < 0.03$) and SD (data not shown pre–post, VF− $P < 0.009$; VF+ $P < 0.05$) were higher during the coronary occlusion after *n*−3 PUFA treatment as compared to values obtained prior to the treatment. However, the absolute change in these variables induced by myocardial ischemia (VF−: ΔHR pre-treatment 23.5 ± 4.3 vs. post-treatment 19.7 ± 4.6 beats/min, ΔHF pre −3.5 ± 0.4 vs. post −2.5 ± 0.5 ln ms^2; ΔSD pre −39.3 ± 10.5 vs. post −25.9 ± 6.5 ms; VF+: ΔHR pre 41.2 ± 5.8 vs. post 35.5 ± 6.8 beats/min, ΔHF pre −4.4 ± 0.5 vs. post −3.6 ± 0.6 ln ms^2; ΔSD pre −39.0 ± 7.9 vs. post −32.8 ± 7.0 ms) was not altered by the *n*−3 PUFA treatment (i.e., there were no significant pre–post × time interactions for either group). Thus, *n*−3 PUFA treatment elicited a similar downward shift in resting HR and an upward shift in the resting

Table 4 | Effect of dietary omega-3 fatty acids on ECG parameters at baseline and during coronary artery occlusion.

	Pre-treatment		Post-treatment	
	Control	**Occlusion**	**Control**	**Occlusion**
HEART RATE (BEATS/MIN)				
VF+				
Placebo	125.6 ± 7.1	172.9 ± 13.6*	124.6 ± 8.4	168.6 ± 16.4*
n−3 PUFA	126.7 ± 5.1	163.9 ± 7.3*	113.9 ± 4.3#	147.3 ± 6.9*#
VF−				
Placebo	122.8 ± 7.2	148.1 ± 12.9*	125.4 ± 9.3	144.7 ± 10.3*
n−3 PUFA	128.6 ± 4.7	146.7 ± 6.0*	116.2 ± 4.1#	139.4 ± 5.9*
PR INTERVAL (ms)				
VF+				
Placebo	91.2 ± 4.9	77.6 ± 6.2*	98.8 ± 5.5	83.8 ± 2.6*
n−3 PUFA	95.3 ± 3.5	87.1 ± 3.5*	104.9 ± 3.4#	96.8 ± 3.5*#
VF−				
Placebo	99.6 ± 3.7	83.6 ± 4.2*	102.3 ± 4.1	99.4 ± 5.0*
n−3 PUFA	98.7 ± 3.4	88.6 ± 3.7*	103.4 ± 3.2	95.4 ± 4.2*
QRS DURATION (ms)				
VF+				
Placebo	77.2 ± 4.3	78.2 ± 4.7	79.6 ± 3.9	79.1 ± 2.1
n−3 PUFA	82.8 ± 1.6	79.0 ± 2.1	82.0 ± 1.1	80.9 ± 1.6
VF−				
Placebo	83.4 ± 2.9	81.1 ± 2.9	88.3 ± 1.6	86.3 ± 2.0
n−3 PUFA	80.0 ± 1.9	79.4 ± 2.0	82.5 ± 1.5	82.1 ± 1.5
QTc INTERVAL (ms)				
VF+				
Placebo	245.6 ± 5.0	248.0 ± 5.2	263.8 ± 5.4#	250.7 ± 5.4
n−3 PUFA	256.2 ± 3.6	257.9 ± 5.8	272.4 ± 3.3#	261.9 ± 3.6#
VF−				
Placebo	251.0 ± 7.5	255.1 ± 5.4	278.0 ± 3.7#	268.3 ± 5.4#
n−3 PUFA	252.4 ± 3.1	248.6 ± 3.2	270.2 ± 3.9#	268.3 ± 4.7#
T_{PEAK}–T_{END} (CORRECTED; ms)				
VF+				
Placebo	89.1 ± 6.2	97.7 ± 6.3*	87.11 ± 3.5	110.3 ± 7.3*
n−3 PUFA	87.7 ± 3.2	103.6 ± 3.7*	89.5 ± 2.9	105.4 ± 4.5*
VF−				
Placebo	86.9 ± 4.5	92.7 ± 5.1	90.7 ± 4.8	91.3 ± 7.1
n−3 PUFA	88.6 ± 3.1	85.6 ± 4.6	89.0 ± 4.5	92.4 ± 4.6

**P < 0.01 control vs. coronary artery occlusion (60 s after occlusion onset); #P < 0.01 Pre-treatment vs. Post-treatment; n−3 PUFA, omega-3 polyunsaturated fatty acids.*

Heart rate correction: QTc = QT − 87(60/HR − 1); T_{peak}–T_{end} (corrected) = T_{peak}–T_{end} − 87(60/HR − 1) (Van de Water et al., 1989); VF+, susceptible to ventricular fibrillation, VF−, resistant to ventricular fibrillation.

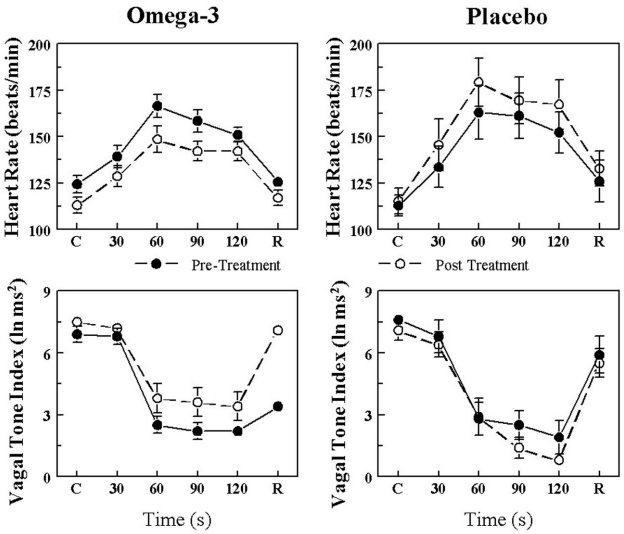

FIGURE 1 | Effect of omega-3 polyunsaturated fatty acids (n−3 PUFAs) on the heart rate and heart rate variability response to acute myocardial ischemia (2 min left circumflex coronary artery occlusion) in dogs susceptible to ventricular fibrillation. Dietary *n*−3 PUFA, but not the placebo, produced a significant downward shift of the heart rate (HR) response that was accompanied by an upward shift in the high frequency (HF) component of the R–R interval variability (vagal tone index 0.24–1.04 Hz; i.e., significant pre–post-treatment effect: HR, $P < 0.02$; HF, $P < 0.03$). Despite the shifts in the curves, the absolute change in these variables induced by the coronary artery occlusion was not altered before or after the treatment (i.e., there were no significant pre–post-treatment × coronary occlusion interactions: HR $P = 0.57$; HF, $P = 0.47$). Placebo ($n = 9$) or *n*−3 PUFA (1–4 g/day, $n = 22$), pre = before treatment began, post = after 3 months of treatment.

HRV in both VF+ and VF− animals but did not alter the magnitude of the change in these variables that was induced by the coronary artery occlusion (i.e., the response to the coronary occlusion *per se* was not affected by the treatment) in either group.

In contrast, the placebo treatment did not alter the HR, HF, or SD (i.e., there were no significant pre–post effects) response to the coronary occlusion in the VF− dogs. In the VF+ dogs, the coronary occlusion elicited larger increases in HR (pre–post, $P < 0.01$) at the end of the 3-month study period, but neither HF nor SD (no significant pre–post effect) was altered by the placebo treatment. As was noted following *n*−3 PUFA treatment, the absolute change in these variables induced by myocardial ischemia was also similar (i.e., there were no significant pre–post × occlusion interactions) in both VF− and VF+ animals before and at the end of the placebo treatment.

EFFECT OF *n*−3 PUFA ON THE HEART RATE AND HEART RATE VARIABILITY RESPONSE TO SUBMAXIMAL EXERCISE

The HR and HRV response to submaximal exercise before and after 3 months of placebo or *n*−3 PUFA treatment are displayed in **Figure 3** (VF+) and **Figure 4** (VF−). As one would predict, exercise provoked large increases in HR (exercise level effect, $P < 10^{-6}$) that were accompanied by large reductions in HRV (exercise level effect, $P < 10^{-6}$) in all four groups. HR was significantly lower (pre–post effect: VF−, $P < 0.05$; VF+, $P < 0.04$), and both HF (pre–post: VF−, $P < 0.02$; VF+, $P < 0.02$) and SD (data not shown pre–post: VF−, $P < 0.0004$; VF+, $P < 0.005$) were higher during exercise after *n*−3 PUFA treatment as compared to values obtained prior to the treatment. In contrast, HR, HF, and SD were not altered (no significant pre–post effects) by the placebo treatment. The change in HR (VF−, pre-treatment 62.4 ± 6.7 vs. post-treatment 66.6 ± 5.5; VF+, pre 65.8 ± 5.3 vs. post 59.8 ± 6.3 beats/min), HF

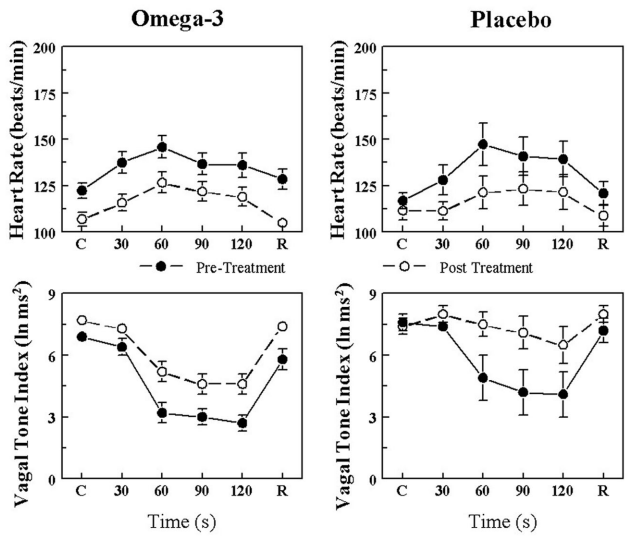

FIGURE 2 | Effect of omega-3 polyunsaturated fatty acids (n−3 PUFAs) on the heart rate and heart rate variability response to acute myocardial ischemia (2 min left circumflex coronary artery occlusion) in dogs resistant to ventricular fibrillation. Dietary *n*−3 PUFA, but not the placebo, produced a significant downward shift of the heart rate (HR) response that was accompanied by an upward shift in the high frequency (HF) component of the R–R interval variability (vagal tone index 0.24–1.04 Hz; i.e., significant pre–post-treatment effect: HR, $P < 0.004$; HF, $P < 0.001$). Despite the shifts in the curves, the absolute change in these variables induced by the coronary artery occlusion was not altered before or after the treatment (i.e., there were no significant pre–post-treatment × coronary occlusion interactions: HR, 0.27; HF, $P = 0.07$). Placebo ($n = 8$) or *n*−3 PUFA (1–4 g/day, $n = 23$), pre = before treatment began, post = after 3 months of treatment.

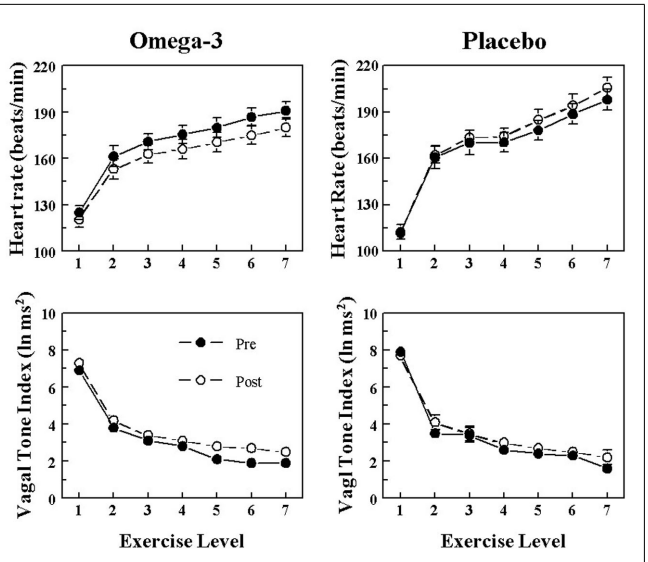

FIGURE 3 | Effect of omega-3 polyunsaturated fatty acids (n−3 PUFAs) on the heart rate and heart rate variability response to submaximal exercise in dogs susceptible to ventricular fibrillation. Dietary *n*−3 PUFA, but not the placebo, produced a significant downward shift of the heart rate (HR) response curve that was accompanied by an upward shift in the high frequency (HF) component of the R–R interval variability (vagal tone index 0.24–1.04 Hz; i.e., significant pre–post effect: HR, $P < 0.04$; HF, $P < 0.02$). Despite the shifts in the curves, the absolute change in these variables induced by the exercise was not altered before or after the treatment (i.e., there were no significant pre–post-treatment × exercise interactions: HR, $P = 0.68$; HF, $P = 0.45$). The data were averaged over the last 30 s of given exercise level. Exercise levels are as follows: 1 = 0 kph and 0% grade; 2 = 4.8 kph and 0% grade; 3 = 6.4 kph and 0% grade; 4 = 6.4 kph and 4% grade; 5 = 6.4 kph and 8% grade; 6 = 6.4 kph and 12% grade; 7 = 6.4 kph and 16% grade. Pre = before placebo ($n = 9$) or *n*−3 PUFA (1–4 g/day, $n = 22$) treatment began, post = after 3 months of treatment.

(VF−, pre -4.9 ± 0.3 vs. post -5.0 ± 0.2; VF+, pre -6.2 ± 0.5 vs. post -6.3 ± 0.5 ln ms^2), and SD (VF−, pre -44.5 ± 4.2 vs. post 60.3 ± 9.3; VF+, -36.5 ± 4.7 vs. post -47.7 ± 6.3 ms) provoked by exercise were not affected by the *n*−3 PUFA treatment (i.e., there were no significant pre–post × exercise level interactions). Thus, *n*−3 PUFA produced a similar shift in the pre-exercise values of HR, HF, and SD in both VF+ and VF− animals and, further, the magnitude of the change in these variables that was induced by exercise was not altered by *n*−3 PUFA for either group. In other words, the response to exercise *per se* was not affected by the treatment.

DISCUSSION

The present study investigated the effects of dietary *n*−3 PUFA (1–4 g/day for 3 months) on HR and HRV (both at baseline and during physiological stress – exercise or acute myocardial ischemia) in dogs with healed myocardial infarction that were known to be either susceptible or resistant to VF. The major findings of the study are as follows: first, and in agreement with previous studies (Harris et al., 2004, 2006; Billman et al., 2010; Billman and Harris, 2011), the *n*−3 PUFA treatment elicited increases in both RBC and cardiac (right atrial and left ventricular) tissue DHA and EPA content. Second, consistent with previous observations (Billman and Harris, 2011) *n*−3 PUFA, but not placebo, treatment elicited reductions in baseline HR, increases in HRV and increases in PR interval. No other ECG parameter was affected by the *n*−3 PUFA treatment. Thus, although the acute application of *n*−3 PUFAs have been shown to decrease action potential duration in isolated cardiomyocytes (Verkerk et al., 2006; den Ruijter et al., 2008, 2010), long-term *n*−3 PUFA treatment did not alter global indices of ventricular repolarization in the present study. Interestingly, QT interval corrected for a HR increased at the end of the 3-month treatment period in both the placebo and the *n*−3 PUFA treated groups, data consistent with a time-dependent electrophysiological remodeling that results as a consequence of myocardial infarction. Third, although the peak values obtained during the stimulus were lower after *n*−3 PUFA treatment as compared to values reached before the treatment began, the absolute magnitude of the change in HR and HRV provoked by either exercise or acute myocardial ischemia was not altered by *n*−3 PUFA treatment and was similar to that recorded for the placebo groups. In other words, the *n*−3 PUFA treatment produced parallel shifts in the response to either exercise or the coronary occlusion due to changes in baseline (pre-challenge) HR and HRV. Fourth, the changes in both the baseline HR and HRV and the changes induced in these variables by a physiological challenge were nearly identical

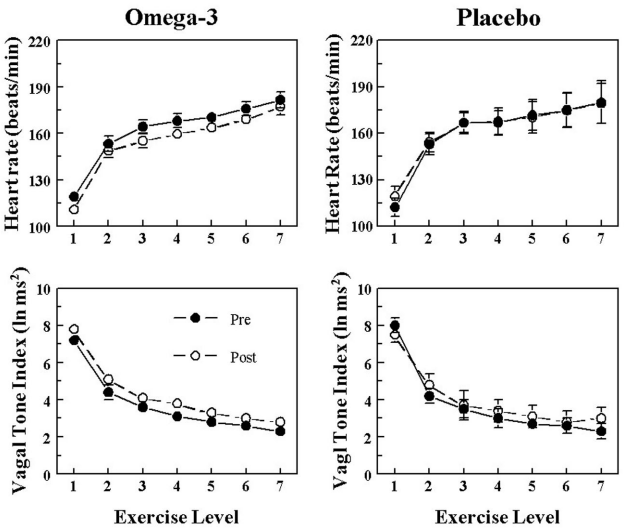

FIGURE 4 | Effect of omega-3 polyunsaturated fatty acids (n−3 PUFAs) on the heart rate and heart rate variability response to submaximal exercise in dogs resistant to ventricular fibrillation. Dietary *n*−3 PUFA, but not the placebo, produced a significant downward shift of the heart rate (HR) response curve that was accompanied by an upward shift in the high frequency (HF) component of the R–R interval variability (vagal tone index 0.24–1.04 Hz; i.e., significant pre–post effect: HR, $P < 0.05$; HF, $P < 0.02$). Despite the shifts in the curves, the absolute change in these variables induced by the exercise was not altered before or after the treatment (i.e., there were no significant pre–post-treatment × exercise interactions: HR, $P = 0.67$; HF, $P = 0.88$). The data were averaged over the last 30 s of given exercise level. Exercise levels are as follows: 1 = 0 kph and 0% grade; 2 = 4.8 kph and 0% grade; 3 = 6.4 kph and 0% grade; 4 = 6.4 kph and 4% grade; 5 = 6.4 kph and 8% grade; 6 = 6.4 kph and 12% grade; 7 = 6.4 kph and 16% grade. Pre = before placebo ($n = 8$) or *n*−3 PUFA (1–4 g/day, $n = 23$) treatment began, post = after 3 months of treatment.

in the susceptible and resistant dogs. These data suggest that despite significantly different initial responses (VF+ animals had a higher HR and lower HRV during exercise or myocardial ischemia as compared to the VF− dogs), *n*−3 PUFA treatment produced similar changes in HR and HRV in dogs that either exhibited VF or had no arrhythmias induced by myocardial ischemia.

EFFECT OF *n*−3 PUFA ON RESTING HEART RATE AND HEART RATE VARIABILITY

In agreement with the present study, a number of clinical (Christensen et al., 1999; Christensen and Schmidt, 2007; Carney et al., 2010; Christensen, 2011) and experimental studies (Laustiola et al., 1986; Kang and Leaf, 1994; Billman et al., 2010; Billman and Harris, 2011; Mayyas et al., 2011) report that *n*−3 PUFA ingestion or acute intravenous administration (Billman et al., 1994) lower HR and increase HRV, suggestive of an increase in cardiac parasympathetic regulation. However, there are also studies in which *n*−3 PUFA failed to alter either HRV or other measures of autonomic function (Russo et al., 1995; Geelen et al., 2003; Hamaad et al., 2006), such as baroreceptor sensitivity (Geelen et al., 2003). Furthermore, even in the studies that reported a positive action of *n*−3 PUFAs on HR or HRV, the effect was often quite small (Mozaffarian et al., 2005, 2006, 2008). For example, a meta-analysis of 30 trials found that fish oil supplements (approximately 3.5 g/day of EPA + DHA) reduced baseline HR by 2.5 beats/min (Mozaffarian et al., 2005), while Mozaffarian et al. (2008) reported that individuals with the highest fish consumption (≥5 meals per weak) only exhibited 1.5 ms greater HRV compared to those with the lowest fish consumption. Although this difference was statistically significant, such a small change in resting HRV is not likely to be physiologically relevant. Indeed, these investigators calculated that only a 1.1% reduction in the relative risk for sudden cardiac death could be associated with this very modest increase in HRV (Mozaffarian et al., 2008). However, these small changes could have important consequences if they are maintained during a physiological stressor such as exercise or acute myocardial ischemia. Reductions in HR would reduce metabolic demand placed on the heart particularly when oxygen supply is compromised by coronary artery lesions/obstructions. The resulting better match between oxygen supply and oxygen demand would, indirectly, decrease the risk for adverse cardiac events associated with myocardial ischemia. In fact, individuals with the lowest resting HRs also exhibited the lowest long-term (>20 years) mortality rate (Jouven et al., 2009). Furthermore, the beneficial effects of beta-adrenergic receptor antagonists, the most effective anti-arrhythmic medication, have been attributed to the negative chronotropic actions of these drugs (Held and Yusuf, 1993). However, it has been recently reported that *n*−3 PUFA treatment not only failed to prevent malignant arrhythmias in VF+ animals, but actually increased ventricular tachyarrhythmias in VF− dogs (Billman et al., 2012). Thus, *n*−3 PUFA mediated reductions in HR were not sufficient to protect against malignant arrhythmias.

EFFECT OF *n*−3 PUFA ON HEART RATE AND HEART VARIABILITY RESPONSE TO MYOCARDIAL ISCHEMIA

As expected from previous studies (Collins and Billman, 1989; Halliwill et al., 1998; Houle and Billman, 1999; Billman, 2006; Billman and Kukielka, 2006), the coronary artery occlusion elicited a robust HR increase that was accompanied by a rapid withdrawal in parasympathetic regulation as indicated by the decline in both total R–R interval variability (SD) and the HF component of R–R interval variability. However, despite alterations in pre-occlusion HR and HRV, the cardiac response to coronary artery occlusion was not altered by the *n*−3 PUFA treatment in either the VF+ or the VF− groups. The *n*−3 PUFA treatment produced parallel shifts in the coronary occlusion response curves (due to changes in the pre-occlusion HR and HRV) but the magnitude of the change in these variables was not altered by the *n*−3 PUFA treatment. As the robust autonomic response to the coronary occlusion was not altered, the changes in pre-ischemic HR and HRV induced by the *n*−3 PUFA may be insufficient to prevent malignant changes in the cardiac rhythm. In fact, as was previously noted, long-term *n*−3 PUFA treatment failed to prevent ischemically induced arrhythmias in the same canine model of sudden cardiac as used in the present study (Billman et al., 2012). These results are analogous to those obtained following treatment with low doses of the cholinergic antagonist atropine (Kottmeier and Gravenstein, 1968; Casadei et al., 1993; De Ferrari et al., 1993; Hull et al., 1995; Halliwill et al., 1998). Using the same canine model of myocardial infarction, low dose atropine decreased baseline HR and increased HRV but,

as in the present study, did not alter the response to myocardial ischemia (Halliwill et al., 1998). This intervention also failed to prevent the induction of VF (Hull et al., 1995; Halliwill et al., 1998). In marked contrast, however, exercise training not only improved resting HR and HRV but also dramatically reduced the response to coronary occlusion and completely suppressed the formation of malignant ventricular tachyarrhythmias (Billman and Kukielka, 2006). When considered together these results strongly suggest that, in order to be effective, an intervention must enhance cardiac parasympathetic regulation during myocardial ischemia; resting changes alone may be insufficient to protect against malignant arrhythmias.

EFFECT OF *n*−3 PUFA ON HEART RATE AND HEART VARIABILITY RESPONSE TO EXERCISE

In agreement with previous human (O'Keefe et al., 2006; Ninio et al., 2008; Peoples et al., 2008; Buckley et al., 2009) and animal studies (Billman and Harris, 2011), dietary *n*−3 PUFA treatment also elicited similar parallel shifts in HR and HRV during exercise but did not alter the response in placebo treated dogs. Furthermore, *n*−3 PUFA provoked nearly identical changes in HR and HRV in both the VF+ and VF− animals. In contrast, an endurance exercise training program (10 weeks treadmill running) both improved resting HR and HRV and attenuated the cardiac response to submaximal exercise in the same canine model as was used in the present study (Billman and Kukielka, 2006, 2007). Unlike *n*−3 PUFA treatment, exercise training also elicited much larger reductions in HR and increases in HRV in VF+ as compared to VF− animals (Billman and Kukielka, 2006, 2007). Thus, exercise training, in marked contrast to *n*−3 PUFA treatment, can elicit larger changes (improvements) in HRV in the animals with more pronounced impairments in cardiac autonomic function than in the dogs with more modest changes in autonomic regulation following myocardial infarction.

LIMITATIONS OF THE STUDY

In the present study cardiac vagal nerve activity was not directly recorded. Cardiac parasympathetic regulation was only indirectly evaluated using non-invasive markers of HRV. Although a number of studies provide strong evidence that beat-to-beat fluctuation in HR reflect corresponding changes in cardiac parasympathetic regulation (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Berntson et al., 1997; Parati et al., 2006; Billman, 2009, 2011), an accurate assessment of nerve activity can only be obtained from direct nerve recordings. As such, HRV data should always be interpreted with care.

Second, previous studies demonstrate that ventricular function is not altered by myocardial infarction in the canine model used in the present study (Billman et al., 1985, 2010; Houle et al., 2001). As such, one might speculate that the potential benefits of dietary *n*−3 PUFA on cardiac autonomic regulation could be more obvious in individuals with more severe cardiac impairment. A more severe impairment in autonomic regulation, particularly during physiological challenges, is consistently noted in VF+ as compared to VF− animals, yet *n*−3 PUFA treatment yielded similar changes in both groups in the present study. Thus, *n*−3 PUFA did not elicit a larger response in the animals with the greatest autonomic impairment. As the effects of *n*−3 PUFA treatment were nearly identical in both VF+ and VF− dogs, these data suggest that perhaps, rather than altering cardiac autonomic regulation, these lipids exert their actions via changes in the intrinsic pacemaker rate. Indeed recent *in vitro* (Verkerk et al., 2009) and *in vivo* (Billman and Harris, 2011) studies demonstrate that *n*−3 PUFA can reduce intrinsic pacemaker rate most likely via action on the pacemaker current (I_f). Further investigation will be required to determine the physiological mechanisms responsible for *n*−3 PUFA mediated changes in HR.

Third, selecting human-equivalent doses of *n*−3 PUFA for animal studies is challenging. The average *n*−3 PUFA dose (adjusted for body surface area) was equivalent to about 5 g/day (VF−, 5.34 ± 0.12 and VF+, 5.17 ± 0.13 g/day) in human subjects. As such, this dose is higher than the 1-g/day dose that has been used in most interventional studies (e.g., Marchioli et al., 2002). However, it is very close to the dose of prescription *n*−3 PUFA (4 g/day, Lovaza®, GlaxoSmithKline) used to treat hypertriglyceridemia (Von Schacky, 2006) and doses up to 8 g/day *n*−3 PUFA have been used to evaluate the effect of *n*−3 PUFA on HRV in human subjects (Peoples et al., 2008). Furthermore, the doses used in the present study yielded RBC membrane EPA + DHA levels that were associated with a significant reduction in the risk for sudden death in epidemiological studies (Siscovick et al., 1995; Albert et al., 2002). Specifically, Albert et al. (2002) found that a mean RBC concentration of 6.9% was associated with a 90% reduction in the risk for sudden death, a value that compares favorably to that obtained in the present study (mean RBC concentration, VF−, $5.5 \pm 0.3\%$ and VF+, $5.4 \pm 0.5\%$, range 2.3–10.7%).

Finally, although dog and man exhibit a similar cardiac autonomic regulation (Scher et al., 1972), species differences could also contribute to response differences. One must always use caution when extrapolating results between species.

CONCLUSION

In the present study, dietary *n*−3 PUFA (DHA + EPA ethyl esters, 1–4 g/day for 3 months) elicited similar reductions in baseline HR that were accompanied by similar increases in HRV in dogs that were susceptible or resistant to VF. However, and in contrast to endurance exercise training (Billman and Kukielka, 2006, 2007), *n*−3 PUFA treatment did not alter the robust autonomic response (identical increases in HR and decreases in HRV before and after treatment) induced by either exercise or, more importantly, by myocardial ischemia in either group of dogs. As both dogs that were either resistant or susceptible to malignant tachyarrhythmias exhibited similar changes in baseline HR and HRV and in the response to physiological challenges, it seems unlikely that changes in HR and HRV are solely responsible for the putative cardiovascular benefits of these lipids.

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The effects of supplementation with omega-3 polyunsaturated fatty acids on cardiac rhythm: anti-arrhythmic, pro-arrhythmic, both or neither? It depends...

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Supplementation of omega-3 fatty acids (Ω-3) has been associated with a decreased cardiovascular risk, thereby concentrating attention on a potentially preventive effect regarding tachyarrhythmias and sudden cardiac death. However, recent randomized controlled trials challenge the efficacy of the additional application of Ω-3 and its anti-arrhythmic effect under certain clinical conditions. The present paper reflects the results of earlier and recent clinical studies with respect to the individual background conditions that may determine the clinical outcome of Ω-3 supplementation and thereby explain apparently conflicting clinical results. It is concluded that the efficacy of Ω-3 supplementation to prevent cardiac arrhythmias strongly depends on the underlying clinical and pharmacological conditions, a hypothesis that also is supported by data from experimental animal studies and by molecular interactions of Ω-3 at the cellular level.

Keywords: omega-3 unsaturated fatty acids, arrhythmia, prevention, cardiovascular disease, myocardial infarction, death, sudden

INTRODUCTION

It is a great desire of humans to find *golden ways* to solve major problems, especially to treat severe diseases effectively in order to prolong life, whenever possible without creating additional risks or side effects. In the past, omega-3 polyunsaturated fatty acids (Ω-3), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), appeared to be compounds having the potential to fulfill this dream, if supplemented to daily nutrition in sufficient amounts.

Consequently, a huge amount of data has been accumulated on this topic and many reviews and meta-analyses have been published (Bucher et al., 2002; Leaf et al., 2003; Whelton et al., 2004; Yzebe and Lievre, 2004; Dhein et al., 2005; Hooper et al., 2006; Reiffel and McDonald, 2006; Wang et al., 2006; Lombardi and Terranova, 2007; Cheng and Santoni, 2008; Jenkins et al., 2008a; León et al., 2008; Siddiqui et al., 2008; Marik and Varon, 2009; Zhao et al., 2009; Filion et al., 2010; Mozaffarian et al., 2011a).

Based on this background the purpose of the present paper is not to again review all the available data on Ω-3 effects or to discuss omega-3 unsaturated fatty acids as essential compounds in human and animal biology. This paper focuses on the effects of supplementation with Ω-3 on cardiac rhythm and discusses the potential clinical consequences of recent clinical studies that do not support the existence of this "golden Ω-3 way". Furthermore, the complexity of the biological interactions of Ω-3 as well as the variation of potential clinical settings are outlined in order to explain that supplementation with Ω-3 does not necessarily result in an overall beneficial clinical effect in every condition.

EARLIER CLINICAL STUDIES

An inverse relationship between consumption of fish oil and cardiovascular risk was shown in early observational, case–control, and cohort studies, with respect to the occurrence of cardiovascular disease (Whelton et al., 2004), sudden cardiac death (SCD) and non-SCD from coronary heart disease (Daviglus et al., 1997), and with regard to SCD in apparently healthy persons (Siscovick et al., 1995; Albert et al., 1998, 2002; Hu et al., 2002; Mozaffarian et al., 2003). Ω-3 levels in erythrocyte membranes were directly associated with a reduced rate of primary cardiac arrest (Siscovick et al., 1995). Similarly, elevated Ω-3 blood levels were associated with a reduced risk of sudden death among men without evidence of prior cardiovascular disease (Albert et al., 2002).

These data were supported by prospective and randomized nutritional intervention studies of secondary prevention after acute myocardial infarction (AMI). In the Diet and Reinfarction Trial (DART) a diet rich in fish and cereals was associated with a significant 29% reduction of all-cause mortality within 2 years after AMI (Burr et al., 1989). In the Lyon Diet Heart Study the Mediterranean diet group [diet enriched by alpha-linolenic acid (ALA, Ω-3) and olive oil, combined with an increased intake of

cereals, fresh fruit, vegetables and fish, but limited intake of saturated fatty acids and linoleic acid (Ω-6)] had a significantly lower rate of the combined endpoint cardiac death and non fatal myocardial infarction, if compared to the control group taking a prudent western-type diet ($p = 0.0001$; follow-up 27 months; de Lorgeril et al., 1994, 1998, 1999).

A predefined supplementation with Ω-3 was used in the large placebo-controlled, open labeled GISSI Prevenzione Trial (EPA + DHA 1 g/day, vitamin E 300 mg/day, a combination of both, or placebo; GISSI-Prevenzione Investigators, 1999), focusing on secondary prevention after AMI. In this study the intervention arms using Ω-3 showed a significant reduction of SCD – though this was not the primary endpoint of the GISSI Prevenzione Trial.

ANIMAL STUDIES

In parallel to these encouraging clinical data, animal studies (mostly using the rat or canine model) supported an anti-arrhythmic effect of Ω-3 especially with respect to ischemia-induced ventricular tachycardia (VT) or ventricular fibrillation (VF) (Matthan et al., 2005; Billman, 2006). The clearest effect in the prevention of VF by Ω-3 could be shown in infusion studies using a special experimental canine model. In this model acute myocardial ischemia was induced at a site distant from a previous myocardial infarction during submaximal exercise thereby activating the autonomic nervous system (Billman, 2006) and inducing VF.

However, these clear effects of Ω-3 under well-defined experimental conditions cannot simply be translated into the clinical situation, and several aspects have to be considered (Billman, 2006):

a. In this canine model not only superfusion with Ω-3 but also the application of β-receptor antagonists, calcium-channel blockers, and endurance exercise training – all interventions that are routinely used in actual clinical practice – were effective in VF prevention.
b. Not all dogs were susceptible to ischemia-induced VF in this model. Animals resistant to VF were characterized by reduced β-receptor responsiveness and an intact parasympathetic regulation, indicating that these are first line mechanisms to prevent ischemia-induced tachyarrhythmias.
c. Finally, incorporation of Ω-3 into the phospholipid bilayer can be expected to be significantly less in infusion studies as compared to feeding studies.

Feeding studies more closely may imitate the clinical situation, and under these conditions Ω-3 can be expected to exert their effect primarily after being incorporated into the cellular membrane. Numerous animal feeding studies have been published between 1987 and 1999, and the results showed a considerable heterogeneity. Still, a meta-analysis of these studies suggests fish oil does prevent ischemia and ischemia-reperfusion induced VT/VF (Matthan et al., 2005).

Heterogeneity of experimental results also can be seen in more recent studies. In isolated hearts of pigs fed with fish oil for 8 weeks, spontaneous ischemia-induced sustained VT/VF was facilitated in the Ω-3 group (Coronel et al., 2007). Other studies report increased resistance to ischemia-reperfusion injury after dietary Ω-3 application, which also could be a basis to protect against reperfusion arrhythmias (Abdukeyum et al., 2008; Zeghichi-Hamri et al., 2010).

RECENT CLINICAL STUDIES

The results of the animal studies and their apparent inconsistencies may be remembered when judging the data of recent prospective, randomized, double-blind clinical studies that interrupted the long list of positive results of older studies investigating the effect of Ω-3 on cardiovascular risk. In the following, these studies and the potential clinical consequences will be discussed in more detail.

1. Three randomized prospective studies evaluating the effect of high doses of Ω-3 in patients with ICD-devices failed to give homogeneous results.

   In one study recurrent VT events not due to myocardial ischemia were more common in patients treated with fish oil (1.3 g Ω-3 per day during a period of two years; $p < 0.007$; Raitt et al., 2005).

   However, in another study predominantly including patients with coronary artery disease, Ω-3 supplementation was associated with a significant risk reduction for the primary endpoint (time to first ICD-event or death from any cause) by 31% ($p = 0.033$). The death rates did not significantly differ between the study groups. Remarkably in this study no significant effect of Ω-3 could be shown in the subgroups of patients without coronary artery disease or with a left ventricular ejection fraction above 30% (Leaf et al., 2005a).

   Finally the SOFA-study did not show a significant effect of Ω-3 supplementation on the primary endpoint (appropriate ICD-interventions for recurrent VT/VF or death from any cause; hazard ratio 0.86, 95% CI 0.64–1.16). The majority of the patients included in the SOFA-study had coronary artery disease, more than 60% with previous myocardial infarction; almost 40% of the study participants had various forms of cardiomyopathy or valvular heart disease (Brouwer et al., 2006).

   In a meta-analysis of these three studies all-cause mortality did not significantly differ between the fish oil and the control groups (relative risk 0.70; 95% CI 0.42–1.15; Jenkins et al., 2008b).

   Finally, in a substudy of the GISSI-HF trial (566 heart failure patients with implanted ICD-devices, 57% with previous myocardial infarction, mean follow-up 928 days) a statistically non-significant trend toward a lower risk of ICD-discharge in patients treated with Ω-3 was shown [adjusted hazard ratio (HR) 0.80; 95% CI 0.59–1.09; $p = 0.152$]. However, mortality was similar in both groups [total mortality: Ω-3 (26.6%), placebo (24.3%); adjusted HR 1.25; 95% CI 0.89–1.75; $p = 0.19$; mortality for arrhythmias: Ω-3 (3.6%), placebo (2.1%); adjusted HR 1.84; 95% CI 0.67–5.05; Finzi et al., 2011]. Therefore the clinical significance of these data remains debatable.

   The apparent heterogeneity in the response of ICD-patients to fish oil supplementation could be a consequence of different study populations and different arrhythmic origins (ischemic versus non-ischemic). Heterogeneity also could be the result

of different concomitant medications of the study populations including β-blockers, digoxin, amiodarone, and sotalol. A potential influence of medication on the effect of fish oil supplementation may be indicated by the results of the DART 2 study (Burr et al., 2003).

2. In the controlled prospective DART 2 trial conducted with general practitioners of South Wales male patients with stable angina ($n = 3{,}114$, under 70 years of age) were randomly allocated to four study groups with specific nutritional advises including the advise to eat oily fish or take fish oil capsules in two of the study groups. Survival was measured during a follow-up of 3–9 years. The risk of SCD was significantly increased in the group taking oily fish or fish oil capsules (HR 1.54; 95% CI 1.06–2.23; Burr et al., 2003). The adverse effects of fish or fish oil capsules only occurred in men not taking beta-blockers or dihydropyridine calcium-channel blockers, and were increased in patients taking digoxin (Burr et al., 2005). Unfortunately, a conclusive interpretation of the DART 2 data is seriously limited as patient's recruitment and monitoring was interrupted for 1 year, long-term compliance was uncertain, and sudden death could not be ascertained in all cases (Burr et al., 2003).
3. In the large scale multicenter Japan EPA Lipid Intervention Study (JELIS; Yokoyama et al., 2007) consumption of 1.8 g EPA per day over a mean period of 4.6 years in hyperlipidemic patients (no AMI in the last 6 months, no serious heart disease) treated with statins resulted in a reduction of major cardiovascular events (combined endpoint including SCD, fatal and non-fatal myocardial infarction, and other non-fatal events including unstable angina, angioplasty, stenting, or coronary bypass grafting) from 3.5 to 2.8% ($p = 0.011$; HR 0.81; 95% CI 0.69–0.95). However, there was neither a reduction of SCD (0.2% in both study arms; HR 1.06; 95% CI 0.55–20.07) nor of coronary death (0.3% in both study arms; HR 0.94; 95% CI 0.57–1.56) or all-cause death (control 2.8%, EPA-group 3.1%; HR 1.09; 95% CI 0.92–1.28). Compared to the GISSI Prevenzione Trial death rates and especially the rates of SCD were very low in both groups and therefore may be difficult to be reduced further by any intervention (rates for SCD: JELIS 0.2% in both groups; GISSI 2.2%/2.9% Ω-3 versus control; GISSI-Prevenzione Investigators, 1999). Furthermore, these low event rates may at least in part be the result of a high fish consumption of the Japanese population at baseline. As most risk reduction already occurs at about 250 mg EPA/DHA intake per day (Mozaffarian and Rimm, 2006), a further increase of Ω-3 intake may not have a substantial additional effect on cardiac death reduction (Mozaffarian, 2007).
4. In the Alpha-Omega-Study, a multicenter, double-blind, placebo-controlled trial, 4,837 patients in the chronic stable phase after myocardial infarction (average 3.7 years after AMI) and 60–80 years of age (21.8% women) were randomly assigned to one of four trial arms. Margarine was used in all trial arms, supplemented with either EPA/DHA (daily intake aimed to be 400 mg), alpha-linolenic acid (ALA, daily intake aimed to be 2 g), EPA/DHA + ALA, or placebo, respectively (Kromhout et al., 2010). After a follow-up of 40 months, 13.9% of the patients had a major cardiovascular event (death, non-fatal cardiovascular events, or cardiac intervention). The rates of the primary endpoint did not differ between the study groups. In addition, in all secondary endpoints, including ventricular-arrhythmia and total death, there was no significant difference between the study groups. Importantly, a high percentage of the patients received "state of the art medication," including statins.

   In a *post hoc* analysis after unblinding of the data in the subgroup of patients with diabetes ventricular-arrhythmia-related events tended to be reduced in the EPA/DHA group (HR 0.51; 95% CI 0.24–1.11) and significantly were reduced in the ALA group (HR 0.39; 95% CI 0.17–0.88). In a secondary analysis of the Alpha-Omega Trial taking high risk patients with previous myocardial infarction and diabetes the EPA/DHA + ALA group experienced significantly less ventricular-arrhythmia-related events (HR 0.16; 95% CI 0.04–0.69; Kromhout et al., 2011). These differential results support the necessity to exactly define the clinical conditions under which supplementation of Ω-3 may be beneficial.
5. In the OMEGA trial the effect of supplementation with 1 g/day of esterified EPA/DHA on the rate of SCD and other clinical events within 1 year after AMI was tested in 3,851 patients (25.6% female, mean age 64.0 years; Rauch et al., 2006, 2010). A 1-year follow-up was chosen, as the risk of cardiac death after AMI including a presumed arrhythmic death is highest in the first 3 months after the event (Solomon et al., 2005; Pouleur et al., 2010). Furthermore, in the GISSI trial, the significance in lowering SCD by Ω-3 had already been reached within 120 days (Marchioli et al., 2002). Following guidelines for the management of AMI and secondary prevention 77% of the patients in the OMEGA trial received acute percutaneous coronary intervention, and/or thrombolysis (8.3%). At hospital discharge the following medications were prescribed for almost all patients: beta-blockers (94%), ACE-inhibitors (83%), ARBs (8%), statins (94%), acetylsalicylic acid (95%), and clopidogrel (88%). Under these conditions, the rates of SCD were 1.5% in both study groups (OR 0.95; 95% CI 0.56–1.60) and total mortality was 4.6% in the Ω-3 group and 3.7% in the control group (OR 1.25; 95% CI 0.90–1.72). In none of the predefined secondary endpoints, including total death, major adverse cardiovascular and cerebrovascular events, and revascularization procedures in survivors, was found a significant difference between the study groups, and not even a trend in favor of the Ω-3 group could be observed. In addition, ICD-terminated VT or VF in survivors was 0.1% ($n = 2/1{,}654$) in the control group but 0.5% ($n = 9/1{,}705$) in the Ω-3 group [$p = 0.06$; OR (95% CI): 4.47 (0.97–20.74)]. Furthermore, there was no significant difference between the study groups with regard to SCD or total death in any of the predefined subgroups of patients with higher risk (diabetes, age >70 years, no acute revascularization, ejection fraction <35%).

   Despite these apparently homogeneous results their interpretation is limited as the case estimate in the OMEGA-study was based on an overestimation of the rate of SCD in the control group, thereby leading to an underpowering of the study.
6. Two other randomized controlled trials published recently also failed to show a clear beneficial effect of Ω-3 supplementation. In 563 elderly Norwegian men at high cardiovascular risk a non-significant tendency to a reduced all-cause mortality

could be observed (HR 0.53; 95% CI 0.27–1.04), but the rate of cardiovascular events remained unchanged (HR 0.89; 95% CI 0.55–1.45, follow-up 3 years; Einvik et al., 2010).

In 2,501 patients with a history of myocardial infarction, unstable angina or ischemic stroke supplementation with EPA/DHA was not associated with a significant decrease of major vascular events during a follow-up of 4.7 years (HR 1.08; 95% CI 0.79–1.47; Galan et al., 2010).

Which conclusions may be drawn from the clinical studies and the animal studies discussed above?

a. The effect of Ω-3 supplementation may depend on the background diet and the pre-existent intake of fish oil (Mozaffarian and Rimm, 2006; Reiffel and McDonald, 2006; Mozaffarian, 2007).
b. With regard to earlier studies, treatment of patients with coronary artery disease, especially treatment of patients with myocardial infarction has improved markedly. In the GISSI trial (inclusion period October 1993 to September 1995) only 4.4% of the patients had acute coronary revascularization at baseline, and only 4.7% were on cholesterol-lowering drugs at hospital discharge, increasing to only 46% after 42 months of follow-up (GISSI-Prevenzione Investigators, 1999). Furthermore, only 43.9% of the patients included in the GISSI trial were on beta-blocker treatment at the start of the study, and this percentage decreased during follow-up. It therefore may be speculated that up-to-date guideline adjusted treatment of AMI (including acute revascularization, medical treatment, and support of life style changes) may interfere with molecular and cellular Ω-3 interactions thereby weakening or competing with a potential beneficial Ω-3 effect. Although the available data not homogeneously support this hypothesis (Marchioli et al., 2007), this aspect should strongly be considered in future research.
c. The anti-arrhythmic effect of Ω-3 may depend on the pathophysiological conditions that facilitate arrhythmias. The clinical and experimental data outlined above suggest that Ω-3 supplementation may especially protect against ischemia-induced arrhythmias. Therefore, prevention of ischemia by modern treatments (i.e., revascularization, beta-blockers, statins, ACE-inhibitors, inhibition of thrombocyte aggregation, physical exercise) could attenuate a potentially beneficial effect of Ω-3. Beta-blockers are well known to prevent sudden death, and even statins could have some anti-arrhythmic effects (Anh and Marine, 2004; Lorenz et al., 2005).
d. Potential anti-arrhythmic effects of Ω-3 by augmentation of vagal activity (Mozaffarian et al., 2005; O'Keefe et al., 2006) may be blunted by beta-blocker treatment and increased physical training during cardiac rehabilitation (Nolan et al., 2008; Billman, 2009).

In summary, the anti-arrhythmic effect proven under experimental conditions in animal models and suggested in the earlier clinical studies appears to depend on the clinical conditions being studied. These clinical conditions are determined by the type and stage of the underlying myocardial disease and represent a sum of various pathophysiological conditions (including ischemia, reperfusion, ischemic preconditioning, scar tissue, inflammation, congenital defects, etc.) and the effects of modern medication including beta-blockers, ACE-inhibitors, statins, and other interventions potentially interfering with the arrhythmic risk, such as exercise training.

These considerations may also apply to the role of Ω-3 in the prevention of atrial fibrillation. Positive results (Mozaffarian et al., 2004, primary prevention in patients >65 years of age; Calò et al., 2005, patients undergoing coronary artery surgery; Macchia et al., 2008, postmyocardial infarction patients) were not confirmed in more recent studies and meta-analyses (Kowey et al., 2010; Saravanan et al., 2010; Bianconi et al., 2011; Farquharson et al., 2011; Liu et al., 2011). Still, it was demonstrated recently, that the use of fish oil (DHA 1.5 g and EPA 0.3 g daily) resulted in a prolongation and reduced dispersion of pulmonary venous and left atrial effective refractory periods in patients with paroxysmal atrial fibrillation (Kumar et al., 2011). Furthermore, in patients with persistent atrial fibrillation on amiodarone and a renin–angiotensin–aldosterone system inhibitor, taking Ω-3 (2 g/day) improved the probability of maintaining sinus rhythm after direct current cardioversion (Nodari et al., 2011). Similar to the prevention of ventricular tachyarrhythmias, prevention of atrial fibrillation therefore may depend on distinct clinical and pathophysiological conditions and concomitant medication. The ongoing OPERA-trial, including a total of 1,516 patients scheduled for cardiac surgery and in sinus rhythm, will give more insight into the potential role of Ω-3 supplementation to prevent post-operative atrial fibrillation (Mozaffarian et al., 2011b).

SOME ASPECTS OF THE MOLECULAR AND CELLULAR INTERACTIONS OF Ω-3 TO EXPLAIN HETEROGENEOUS CLINICAL RESULTS

For understanding the seemingly heterogeneous efficacy of Ω-3 supplementation in preventing tachyarrhythmias, it is important to reflect on their molecular and cellular interactions as has been delineated extensively in recent reviews (Leaf et al., 2003, 2005b; Dhein et al., 2005; McLennan and Abeywardena, 2005; Den Ruijter et al., 2007; Lombardi and Terranova, 2007; Siddiqui et al., 2008). In the following, only some aspects of potential relevance for interpretation of the clinical data are discussed:

There are three major ways in which Ω-3 may interfere with cellular and membrane function, thereby potentially moderating cardiac rhythm:

a. Direct interactions of Ω-3 with membrane bound proteins like the fast sodium channel, the voltage-gated L-type Ca^{2+} channel, specific potassium channels, and the Na^{+}/Ca^{++}-exchanger (Hallaq et al., 1990, 1992; Honore et al., 1994; Xiao et al., 1995, 1997; Kang and Leaf, 1996; Leifert et al., 1999; Leaf et al., 2003; Den Ruijter et al., 2007; Wang et al., 2010). Such interactions may occur predominantly with circulating Ω-3 when it is delivered by acute administration and infusion.
b. Incorporation into the phospholipid bilayer, thereby potentially changing membrane fluidity, and/or forming Ω-3 rich microdomains, and/or interacting with internal binding sites. This may result in a change of the function of membrane bound proteins like ion channels, receptors and signal transduction

systems (McMurchie et al., 1988; Croset and Kinsella, 1989; Kinoshita et al., 1994; Grynberg et al., 1996; Leifert et al., 1999, 2000b; McLennan, 2001; Den Ruijter et al., 2007). Incorporation into the cellular membranes predominantly is achieved by dietary long-term administration of Ω-3.

c. Interaction with intracellular pathways including gene expression and metabolism of phosphoinositides (Judé et al., 2006).

Circulating Ω-3 compounds are likely to have different electrophysiological effects, compared to Ω-3 incorporated into the membranes (Den Ruijter et al., 2007 for review). For example, peak cardiac sodium current was reduced by 51% after acute administration of EPA and DHA in neonatal rat cardiomyocytes (Xiao et al., 1995), but remained unaffected by Ω-3 incorporated in pig and rat cardiomyocytes (Leifert et al., 2000a; Verkerk et al., 2006). Differential effects of circulating versus incorporated Ω-3 have also been demonstrated with respect to various potassium channels and the regulation of calcium homeostasis (Den Ruijter et al., 2007 for review). Incorporated Ω-3, however, also may prevent further action potential (AP) shortening induced by circulating Ω-3. Patients with high levels of incorporated Ω-3 therefore may not have a further benefit from short term Ω-3 supplementation (Den Ruijter et al., 2010). This could be of a direct clinical relevance, as acute Ω-3 supplementation may be used for prevention of atrial fibrillation induced by cardiac surgery, which is being investigated in the OPERA-trial (Mozaffarian et al., 2011b).

Apart from these considerations the molecular interactions of Ω-3 and their effects on cardiac rhythm may be influenced by a large variety of additional conditions:

a. The various kinds of Ω-3 formulations being used (re-esterified triacylglycerides, ethyl-esters or phospholipids; Neubronner et al., 2011; Schuchardt et al., 2011).
b. The activity state of membrane bound proteins and ligand occupation of specific receptors involved in signal transduction (Rauch et al., 1989; Xiao et al., 1998; Den Ruijter et al., 2007), or the increased responsiveness of inhibitory G-proteins after ischemic preconditioning (Niroomand et al., 1995).
c. The activity of cellular phospholipases and the presence of lysophosphatides that change phospholipid environment and function of membrane bound proteins (Chien et al., 1981; Corr et al., 1984; Rauch et al., 1994), and may even vary between different myocardial regions depending on the degree of ischemia and/or inflammation.
d. The heterogeneity of electrical stability of myocardial cells in the diseased heart muscle due to regional differences with regard to various degrees of ischemia and tissue damage, ischemic preconditioning, etc. (Dhein et al., 2005). In this respect it should also be remembered, that in patients with coronary artery disease, myocardium is not presenting as a homogeneous and healthy tissue experiencing acute ischemia in a well-defined area, but rather as a mixture of healthy myocardium, hypertrophied tissue, scar tissue, and ischemic myocardium and includes areas of tissue with ischemic preconditioning, inflammation, various degrees of membrane phospholipid degradation and with more or less acute or chronic stretch, etc. (Janse et al., 2003).
e. The species (human, various animals) being studied. The characteristics of APs vary significantly between human and various animal myocardial cells and with gender (Karagueuzian et al., 1982; Shattock and Bers, 1989; Cheng, 2006; Tanaka et al., 2008).
f. The various mechanisms that trigger VT and VF. Under clinical conditions VT or VF are predominantly caused by triggered activity or by re-entry mechanisms. Fish oil shortens cardiac AP and accentuates the AP notch, which may lead to depression or even loss of the AP dome (Verkerk et al., 2006, 2007). Under clinical conditions where the AP is prolonged triggered activity may be the predominant pro-arrhythmic mechanism, which could be inhibited in isolated cardiomyocytes from rabbits and from patients with end stage heart failure by superfusion with Ω-3 (Den Ruijter et al., 2008). Triggered activity also could be inhibited in pig cardiomyocytes (Den Ruijter et al., 2006). In keeping with these experimental results Ω-3 were effective in reducing the arrhythmic risk in patients with idiopathic dilated cardiomyopathy (Nodari et al., 2009).
Conversely, AP shortening also may be pro-arrhythmic by reducing the refractory period and thereby promoting re-entry. Supplementation with Ω-3 may increase a preexisting heterogeneity in AP duration and repolarization (Verkerk et al., 2007), as can be seen, for example, in acute ischemia (Yan et al., 2004). In this way the occurrence of unidirectional block and re-entry may be facilitated (Janse and Wit, 1989). In the clinical situation therefore supplementation with Ω-3 may prevent or facilitate ventricular tachyarrhythmias depending on the predominant underlying arrhythmic mechanism (Den Ruijter et al., 2007).

Based on these considerations it becomes apparent that Ω-3 do not have a specific way to act, but rather possess multiple sites of potential actions, that may be influenced by a number of external conditions at the cellular and molecular level. Multiple sites of interaction between Ω-3 and myocardial tissue in combination with various possible ways of interference with these biochemical interactions are unlikely to result in an unequivocally predictable and homogeneous beneficial effect on clinical outcomes.

CONCLUDING REMARKS

Ω-3 clearly interfere with the physiology of myocardial cell membranes through a variety of specific and unspecific pathways, and thereby exhibit anti-arrhythmic effects under certain well-defined experimental and clinical conditions. However, these membrane effects of Ω-3 are complex. This complexity makes it difficult to predict the effects of Ω-3 supplementation on cardiac rhythm within the wide variety of conditions that represent clinical practice. For the future it will be necessary to define exactly the clinical conditions in which supplementation with of Ω-3 is beneficial, and without potentially harmful effects.

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Omega-3 fatty acids: anti-arrhythmic, pro-arrhythmic, or both?

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This review focuses on developments after 2008, when the topic was last reviewed by the author. Pertinent publications were found by medline searches and in the author's personal data base. Prevention of atrial fibrillation (AF) was investigated in a number of trials, sparked by one positive report on the effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), considerations of upstream therapy, data from electrophysiologic laboratories and animal experiments. If EPA + DHA prevent postoperative AF, the effect is probably smaller than initially expected. The same is probably true for maintenance of sinus rhythm after cardioversion and for new-onset AF. Larger trials are currently ongoing. Prevention of ventricular arrhythmias was studied in carriers of an implanted cardioverter-defibrillator, with no clear results. This might have been due to a broad definition of the primary endpoint, including any ventricular arrhythmia and any action of the device. Epidemiologic studies support the contention that high levels of EPA + DHA prevent sudden cardiac death (SCD). However, since SCD is a rare occurrence, it is difficult to conduct an adequately powered trial. In patients with congestive heart failure, EPA + DHA reduced total mortality and rehospitalizations, but not SCD or presumed arrhythmic death. Of three trials in patients after a myocardial infarction, two were inadequately powered, and in one, the dose might have been too low. Taken together, while epidemiologic studies support an inverse relation between EPA + DHA and occurrence of SCD or arrhythmic death, demonstrating this effect in intervention trials remained elusive so far. A pro-arrhythmic effect of EPA + DHA has not been seen in intervention studies, and results of epidemiologic and animal studies also rather argue against such an effect. A different, and probably more productive, perspective is provided by a standardized analytical assessment of a person's status in EPA + DHA by use of the omega-3 index, EPA + DHA in red cell fatty acids. In populations with a high omega-3 index, SCD is rare. Intervention trials can become more effective by including a low omega-3 index into the inclusion criteria, thus creating a study population more likely to demonstrate an effect of EPA + DHA. This is especially relevant in case of rare endpoints, like new-onset AF or SCD.

Keywords: eicosapentaenoic acid, docosahexaenoic acid, omega-3 fatty acids, omega-3 index, atrial fibrillation, ventricular tachycardia, ventricular fibrillation, sudden cardiac death

INTRODUCTION

The view that the two marine omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) impact on cardiac rhythm goes back a number of years, and has been reviewed by this author in 2008 (von Schacky, 2008). Underlying mechanisms have recently been reviewed (Richardson et al., 2011). The impression that EPA + DHA are primarily anti-arrhythmic has been challenged by results of clinical studies, reporting that EPA and DHA did not reduce sudden cardiac death (SCD; Rauch et al., 2010). Moreover, in one study, it had been reported that higher levels of EPA and DHA in red blood cells were associated with a higher likelihood of ventricular arrhythmias (Wilhelm et al., 2008). These results are in contrast to the manifold evidence present already in 2008, indicating anti-arrhythmic effects of EPA and DHA (von Schacky, 2008). The present review will highlight recent developments and put them into perspective.

METHOD

Medline searches were performed with the combination of "omega-3," "*n*−3 fatty acids" "eicosapentaenoic," or "docosahexaenoic" with "arrhythmia," and investigations conducted in humans and fully published in more recent years than 2008 were selected. Studies on animals were also included, in case animals were pre-fed with omega-3 fatty acids. In addition, the author's personal base of publications was used. Abstracts presented at scientific meetings were not included.

Abbreviations: AF, atrial fibrillation; CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HR, hazard ratio; ICD, implanted cardioverter-defibrillator; SCD, sudden cardiac death; VF, ventricular fibrillation; VT, ventricular tachycardia.

ANIMAL STUDIES

Experiments on ion channels in cultured cells, on cardiac tissue *in vitro*, Langendorff preparations of explanted hearts, or using similar techniques investigating acute effects of EPA and/or DHA are reviewed elsewhere (De Caterina, 2011; Mozaffarian and Wu, 2011; Richardson et al., 2011; Savelieva et al., 2011). Of note, in such studies biologically active metabolites of EPA and DHA were defined, making the developments of more effective compounds a distinct possibility (e.g., Falck et al., 2011).

CARDIAC RHYTHM

Heart rate and heart rate variability were recorded at rest, during submaximal exercise, and during a 2-min coronary artery occlusion at rest in dogs with a healed myocardial infarction before and after 3 months supplementation with a fish oil or corn oil. Fish oil, but not corn oil reduced heart rate and heart variability at rest, but not during exercise or during coronary occlusion (Billman and Harris, 2011).

NEW-ONSET POSTOPERATIVE AF

Dogs were fed a fish oil-enriched chow (or control) for 3 weeks before excision of the left atrial appendage. Burst pacing induced atrial fibrillation (AF) only in control dogs, whereas postoperative atrial effective refractory period was prolonged, heart rate was lower, and heart rate variability higher in the fish oil-fed dogs. Less postoperative inflammatory reaction was observed in the atria of fish oil-fed dogs (Mayyas et al., 2011).

NEW-ONSET AF

After 7 days feeding of a fish oil or a control chow, dogs were atrioventricularly paced at 220 bpm for 14 days (feeding was continued). A third group of dogs was fed a regular chow and was not paced. Atrial tissue was sampled and analyzed for gene expression using a quantitative reverse real-time polymerase chain reaction. Genes related to fibrosis, hypertrophy, and inflammation were found to be down-regulated by the fish oil supplement (Ramadeen et al., 2010). More recently, the same group initiated treatment with EPA + DHA 7 days after simultaneous atrial and ventricular pacing was initiated to induce AF (Ramadeen et al., 2012). After a total of 14 days of pacing, no effects of EPA + DHA were seen on development of AF, echocardiographic and histologic parameters, and on expression of fibrosis-related genes, which was in contrast to the previous study, where dogs were fed before pacing (Ramadeen et al., 2012). In a similar study, after 7 days of feeding EPA, rabbits were subjected to ventricular pacing at 400 bpm for 4 weeks to induce heart failure (feeding was continued). One group of control rabbits was not fed EPA, one group was not paced. Duration of AF after burst pacing was attenuated in the EPA group, as was atrial fibrosis and a number of inflammatory parameters (Kitamura et al., 2011).

Taken together, these animal studies indicate that the propensity develop AF is mitigated by pre-feeding omega-3 fatty acids by interactions at various levels – from gene expression to refractory period. However, use of EPA + DHA 7 days after initiation of pacing had no effect. No study was found indicating a pro-arrhythmic effect on the supraventricular level.

VENTRICULAR ARRHYTHMIAS/CELL PREPARATIONS

Infusion of omega-3 fatty acids corrected some, but not all abnormalities of ventricular ion channel function induced by omega-3 depletion in rats (Peltier et al., 2009).

VENTRICULAR ARRHYTHMIAS/LANGENDORFF PREPARATIONS

Rats were fed diets enriched in fish oil, sunflower oil, or beef tallow for 6 weeks. The hearts were either subjected to ischemic preconditioning or not. It was found that ischemic preconditioning reduced ventricular arrhythmias in all dietary groups, but mostly so in the fish oil group (Abdukeyum et al., 2008). In another study, rabbits were fed fish oil or a control laboratory chow for 30 days. The hearts were challenged with dofetilide, a selective rapidly activating delayed rectifier potassium current inhibitor with pro-arrhythmic effects. Dofetilide-induced triangulation, reverse use-dependence, instability, and dispersion were reduced and torsade de pointes abolished (Dujardin et al., 2008). Pre-feeding with omega-3 fatty acids did not suppress the incidence of ventricular reperfusion arrhythmias after global no-flow ischemia in hearts of female mice (Huggins et al., 2009). Rats with or without hypertriglyceridemia were given atorvastatin and/or omega-3 fatty acids for 2 months. Threshold for VF was tested by electrical stimuli. Threshold for VF was lower in hypertriglyceridemic rats, but was significantly increased by omega-3 fatty acids, an effect found to be related to an improvement in cell-to-cell junction integrity (Bacova et al., 2010).

VENTRICULAR ARRHYTHMIAS/WHOLE ANIMAL EXPERIMENTS

Pericardial infusion of DHA reduced infarct size and ventricular arrhythmias in pigs subjected to experimental myocardial infarction (Xiao et al., 2008). Cells from explanted hearts from rabbits or humans with congestive heart failure were superfused with EPA or DHA. This abolished triggered activity and reduced the number of delayed afterdepolarizations and calcium aftertransients compared with control and oleic acid (Den Ruijter et al., 2008). In aged spontaneously hypertensive rats, fish oil feeding for 2 months suppressed inducible ventricular fibrillations, and improved other cardiac parameters in comparison to untreated controls (Mitasíková et al., 2008). Dogs were subjected to an experimental myocardial infarction, and 4 weeks later randomized to receive omega-3 fatty acids or placebo for 3 months. Ventricular function was then assessed in a number of ways, but no differences were found (Billman et al., 2010). Size of an experimental infarction was smaller in rats given omega-3 fatty acids than diets rich in saturated or omega-6 fatty acids (Zeghichi-Hamri et al., 2010). Male pigs were fed EPA or a control chow for 3 weeks, and then a 90-min myocardial ischemia was induced. EPA attenuated occurrence of VF, reduced mortality, and attenuated monophasic action potential duration shortening during ischemia (Tsuburaya et al., 2011). Very recently, results of experiments in dogs with healed myocardial infarction, made ischemic by occlusion of the circumflex artery in the last minute of exercise were reported (Billman et al., 2012). The dogs received either a corn oil placebo or doses of EPA + DHA ranging from 1 to 4 g/day for 3 months, the latter being incorporated into red cell and cardiac tissue fatty acids. EPA + DHA did not prevent arrhythmias in dogs in which VF could be induced before treatment started (decreased in 27%

placebo vs. 24% $n-3$ PUFA, $p = 0.5646$) but made dogs in which VT or VF could not be induced before treatment susceptible ($n-3$ PUFA 33% vs. placebo 0%, $p = 0.0442$). The authors concluded that dietary $n-3$ PUFAs did not prevent ischemia-induced VF and actually increased arrhythmia susceptibility in both non-infarcted and low-risk post-MI dogs (Billman et al., 2012).

Taken together, pre-feeding omega-3 fatty acids before experimental myocardial infarctions reduced mortality, infarct size and ventricular arrhythmias. However, 4 weeks after an experimental myocardial infarction, feeding omega-3 fatty acids did not affect ventricular function, and reduced VT's in two, and increased VT's in one study. Most studies of Langendorff preparations of hearts from animals pre-fed with omega-3 fatty acids indicate a reduced propensity toward ventricular arrhythmias. One study saw no suppression of reperfusion arrhythmias. Taken together, results from animal studies are not homogeneous, and might depend on the model studied. In this authors personal opinion, results from animal studies should have little impact on clinical decisions. Rather, clinical decisions should be guided by data derived from human studies, preferably large intervention trials.

STUDIES IN HUMANS

SUPRAVENTRICULAR ARRHYTHMIAS

Aside from epidemiologic observations (Gronroos and Alonso, 2010; Wu et al., 2012), promising results of studies on effects of EPA and DHA on atrial tissue or cells, and a number of animal models mentioned above, and more extensively reviewed in Savelieva et al. (2011), interest in randomized clinical trials was sparked by a trial, in which EPA and DHA prevented new-onset AF in patients after a coronary bypass operation (Calò et al., 2005). Supportive evidence was provided by a small randomized controlled intervention trial on parameters measured in the electrophysiology laboratory in a total of 36 patients, demonstrating that a mean treatment of 40 days with 1.8 g/day EPA + DHA in a fish oil prolonged pulmonary venous and left atrial effective refractory periods and decreased susceptibility to initiation AF from within pulmonary veins (Kumar et al., 2011a). In two follow-up studies from the same group, using the same approach, it was found that 1.8 g/day EPA + DHA prolonged atrial refractoriness and reduced vulnerability to inducible AF, and attenuated atrial mechanical stunning after reversion of atrial arrhythmias to sinus rhythm (Kumar et al., 2011b,c). While the results just mentioned were promising, they needed to be scrutinized in trials using occurrence of AF as an endpoint.

New-onset postoperative AF

For a randomized double-blind single-center study in Germany, 102 patients were recruited before a coronary bypass operation (Heidt et al., 2009). Throughout the hospital stay, they were given 1 ml/kg body weight/day of an intravenous formulation. In the intervention group of 52 patients, this formulation contained, according to the authors, 10 mg of a fish oil per 100 ml, which, again according to the authors, resulted in a dose of 100 mg fish oil/kg body weight/day. According to a reference manual, 100 ml of this formulation contain between 1.25–2.82 g EPA and 1.44–3.09 g DHA (http://new.ch.oddb.org/de/drugs/fachinfo/uid/186817). The control group received a soy-based intravenous formulation, containing 10 g of soy bean oil per 100 ml, resulting in 100 mg soy bean oil/kg body weight/day. Sample size calculation was based on a 30% occurrence of postoperative AF in the control group and a 20% occurrence in the intervention group, and testing for significance after each event (but no correction for multiple testing), other assumptions were not reported. Heart rhythm was monitored continuously on the intensive care unit. It is not reported, whether all or how many of the recruited patients were evaluated at study end. According to table 3 of the publication, 3 days postoperatively, 11 patients in the control group (according to the authors 11.58%), and 7 patients in the intervention group (according to the authors 7.37%) developed AF. According to the authors, "after the 18th test, the procedure can be stopped with a probability of error of 0.01." In the abstract of the publication, the authors state: "Postoperative AF occurred in 15 patients (according to the authors 30.6%) in the control and in 9 (according to the authors 17.3%) in the PUFA group ($p < 0.05$)." In two figures, a trend toward a shorter hospital stay for patients in sinus rhythm is depicted, but in the abstract, the authors state: "After CABG, the PUFA patients had to be treated in the ICU for a shorter time than the control patients." The authors concluded that "perioperative intravenous infusion of PUFA should be recommended for patients undergoing CABG," because "PUFA reduces the incidence of AF after CABG and leads to a shorter stay in the ICU and in hospital." The latter conclusion does not appear to be supported by a significant difference in hospital stays between control and intervention groups, and the former is based on numbers that are difficult to comprehend.

For another randomized double-blind single-center study, this time in the UK, 108 patients were recruited before a coronary bypass operation (Saravanan et al., 2010). Patients were recruited 1–3 weeks preoperatively, and were given either two 1 g capsules per day of a 85% fish oil concentrate in the form of an ethyl-ester (54 patients), or identical capsules containing olive oil as a placebo (54 patients). Sample size was calculated based on an expected incidence of postoperative AF of 50% in the placebo group and a relative risk reduction of 55% by EPA and DHA, and the usual assumptions ($\alpha < 0.05$, $\beta = 0.8$). Heart rhythm was monitored continuously with a recorder for 5 days postoperatively, and episodes of AF >30 s duration were considered. At study end, in the intervention group, 52 patients were analyzed, and 51 in the placebo group. Mean treatment duration was 17 days, and AF developed in 43% of the placebo group, and 56% of the intervention group (n.s.). Hospital stay and other parameters were also not significantly different. The authors concluded that "omega-3 PUFA do not reduce the risk of AF after coronary artery bypass graft surgery" (Saravanan et al., 2010).

A similar, randomized double-blind single-center trial was conducted in Iceland, for which 170 patients were recruited (Heidarsdottir et al., 2010). Patients were started 5–7 days preoperatively either on two soft capsules daily, totaling either 1240 mg of EPA and 1000 mg DHA as ethyl-esters (84 patients), or olive oil as a placebo (86 patients). Sample size was calculated based on an expected incidence of postoperative AF of 40% in the placebo group, a reduction to 20% by the intervention, and the usual assumptions. At study end, 83 patients were evaluated in the intervention

group, and 85 in the placebo group. Onset of AF of a duration of >5 min was assessed by continuous electrographic monitoring. Treatment duration was 2–28 days, and postoperative AF was observed in 54.2% in the intervention group and in 54.1% of the placebo group (n.s.), although EPA and DHA were significantly higher in plasma phospholipids in the intervention group than in the control group ($p < 0.01$). Other outcomes were not significantly different, including safety parameters. The authors concluded that "There is no evidence for a beneficial effect of treatment with $n-3$ PUFA on the occurrence of postoperative AF in patients undergoing open heart surgery" (Heidarsdottir et al., 2010). In a substudy in the 140 patients undergoing coronary bypass grafting, it was found that EPA and DHA might prevent AF in patients with low plasma phospholipid baseline levels of EPA and DHA, but might be harmful in those with high levels (Skuladottir et al., 2011). These findings, however, were not very robust.

In an observatory study in 530 Italian patients undergoing cardiac surgery, preoperative intake of 860 mg EPA + DHA ethyl-ester for a median of 5 days by 16% of the population was associated with a decreased incidence of early, but not late AF after cardiac surgery (Mariscalco et al., 2010).

In Australia, for a single-center randomized controlled, double-blind study, 200 patients were randomized after a coronary bypass or cardiac valve operation (Farquharson et al., 2011). A fish oil, providing a daily dose of 4.5 g EPA + DHA was compared to another oil, largely containing oleic acid, in the form of 15 ml liquid, which was to be taken from 3 weeks pre-operatively on. The primary outcome was occurrence of sustained AF/atrial flutter (duration ≥10 min or requiring intervention) during the first 6 postoperative days. Sample size was calculated based on an expected incidence of postoperative AF in 42% of patients, a 53% relative risk reduction and the usual assumptions. An intention-to-treat analysis was performed in the 194 participants operated upon. Onset of AF was assessed based on continuous electrocardiographic monitoring. In the intervention group, EPA and DHA increased in red cells from 5.91 ± 1.10 to $8.80 \pm 1.73\%$ ($p < 0.05$), but remained stable in the control group. AF occurred in 47 of 97 (48%) in the control group and 36 of 97 (37%) in the fish oil group ($p = 0.11$). Time to AF was non-significantly longer ($p = 0.06$), and stay in the ICU significantly shorter ($p = 0.005$) in the intervention group, adverse events were evenly distributed. The authors concluded that "dietary fish oil did not result in a significant decrease in the incidence of postsurgical AF," but noted the significant decrease in time spent in the intensive care unit (Farquharson et al., 2011).

A systematic review on trials on prevention of new-onset postoperative AF that did not include the Australian trial, noted non-significant reductions in the occurrence of postoperative AF of 22% (OR, 95% CI 0.48–1.27), and called for larger trials (Liu et al., 2011). One larger trial is currently ongoing (Mozaffarian et al., 2011b). Whether new-onset postoperative AF can be considered a model for new-onset of AF without a prior cardiac operation remains a matter of debate. However, as demonstrated by the trials discussed above, new-onset postoperative AF is a common and unresolved problem after cardiac surgery, and any form of effective low-risk prevention would be welcome.

New-onset of AF

In an observatory study, based on an Italian population of 2,239,205 subjects, using hospital discharge records, prescription databases, and the civil registry, patients discharged after an acute myocardial infarction free of AF, were monitored for 360 days for death from any cause and for new-onset AF (Macchia et al., 2008). Those prescribed EPA + DHA had a relative risk for hospitalization for AF of 0.19 (hazard ratio, 95% CI 0.07–0.51], and for all-cause mortality of 0.15 (hazard ratio of 95% CI 0.05–0.46). Another observatory study in 2174 Finnish subjects, followed for 17.7 years, 240 developed new AF (Virtanen et al., 2009). The quartile with the highest serum DHA had a relative risk for new AF of 0.64 (hazard ratio, 95% CI 0.42–0.92). A publication from the Women's Health Initiative saw no relation between fish intake and new-onset of AF in 44,720 participants, subjected to electrocardiograms after 3 and 6 years (Berry et al., 2010). In contrast, in an Italian study, the red cells of 40 patients with AF/atrial flutter contained a higher percentage of EPA and DHA than those of 53 controls (Viviani Anselmi et al., 2010). In keeping, a report from the Framingham study saw an increased risk for incident AF with >4 servings of dark fish (Shen et al., 2011). Clearly, to define the role of EPA and DHA in prevention of new-onset of AF, a large scale clinical trial is required. The author is part of a world-wide effort to make such a trial possible.

Prevention of recurrent AF

In an observational study on 1500 patients undergoing catheter ablation, 285 were treated with omega-3 fatty acids (Patel et al., 2010). Of those 129 were matched to 129 controls. Of the patients treated with omega-3 fatty acids, 35 (27.1%) had early recurrence vs. 57 (44.1%) in the control group ($p < 0.0001$). Less procedural failures were observed in the treated group ($p < 0.003$; Patel et al., 2010).

A multi-center randomized controlled double-blind 24-week trial was conducted in 663 US outpatient participants with confirmed symptomatic paroxysmal ($n = 542$) or persistent ($n = 121$) AF (Kowey et al., 2010). Participants received either four 1 g capsules per day of an 85% fish oil concentrate in the form of an ethyl-ester (332 patients), or identical capsules containing olive oil as a placebo (331 patients). The primary end point was a first symptomatic recurrence of AF in participants with paroxysmal AF. Study size was based on a 32% risk reduction, and the trial was to be stopped at 295 primary endpoints (Pratt et al., 2009). Five hundred eighty-four participants completed the study. Data were analyzed according to modified intention-to-treat. Although EPA and DHA increased in plasma, no differences were noted in the primary endpoint: in participants with paroxysmal AF (hazard ratio, 1.15; 95% CI 0.90–1.46; $p = 0.26$), or in participants with persistent AF (HR, 1.64; 95% CI 0.92–2.92; $p = 0.09$), or both combined (HR, 1.22; 95% CI 0.98–1.52; $p = 0.08$). Secondary endpoints also did not differ. Heart rate during the recurrent AF was lower in the group with EPA + DHA by 6.88 bpm than in the control group. Untoward effects were evenly distributed. The authors concluded that "Among participants with paroxysmal AF, 24-week treatment with prescription omega-3 compared with placebo did not reduce recurrent AF over 6 months" (Pratt et al., 2009; Kowey et al., 2010).

Whether three 1 g capsules of an 85% EPA + DHA ethyl-ester would prevent recurrence of AF after electric cardioversion was tested in a multi-center randomized controlled double-blind 6 month trial in Italy vs. an olive oil placebo (Bianconi et al., 2011). Patients were included, if AF had persisted >1 month. The primary endpoint was the percentage of recurrence, and study sized was based on a recurrence of 50% in control patients, and 35% in intervention patients, and the usual assumptions. Into the intervention group, 111 patients were recruited, and 103 into the placebo group. Included into the analysis were 104 patients (intervention) and 100 (placebo). EPA and DHA increased significantly in the intervention group, but remained stable in the control group. Sinus rhythm was restored, either spontaneously or after electric cardioversion, in 187 patients (91.7%); 95 patients (91.4%) on EPA + DHA, and 92 patients (92.0%) on placebo (p = n.s.). AF relapsed in 56 (58.9%) on EPA + DHA and in 47 (51.1%) of the placebo patients (p = n.s.). The mean time to AF recurrence was 83 + 8 days in the EPA + DHA group and 106 + 9 days in the placebo group (p = n.s.). Adverse events were evenly distributed. The authors concluded that their "results do not support the hypothesis that in patients undergoing electric cardioversion of chronic persistent AF, supplementation with PUFAs in addition to the usual anti-arrhythmic treatment reduces recurrent AF" (Bianconi et al., 2011).

Very recently, a similar randomized controlled double-blind single-center trial was published from Italy (Nodari et al., 2011). Two 1 g capsules contained either a 85% EPA + DHA ethyl-ester or an olive oil placebo. Patients with persistent AF were included, if they had at least one relapse after a previous cardioversion, had an atrial size of >60 mm in a echocardiogram, but all were treated with amiodarone and an inhibitor of the renin–angiotensin system. Patients were pre-treated for 4 weeks before electric cardioversion. The primary endpoint was the probability of AF after 1 year. Study size was estimated based on a relapse rate of 50%, a 20% reduction of that rate by the intervention and the usual assumptions. Into the intervention group, 102, into the placebo group 103 patients were recruited, and in both groups 94 patients completed the study, but the analysis was intention-to-treat. The probability of maintenance of sinus rhythm was higher in the intervention group than in the placebo group (HR 0.62, 95% CI 0.52–0.72, $p < 0.0001$). Adverse effects were minor in both groups. The authors concluded that EPA + DHA may exert beneficial effects in the prevention of AF recurrence," and state that "Further studies are needed to confirm and expand our findings" (Nodari et al., 2011).

During writing of this review, results of an open, uncontrolled single-center intervention were reported (Watanabe et al., 2011). For 6 months, patients with paroxysmal AF were on either propafenone or flecainide, and for the subsequent 6 months 1.8 g EPA-ethyl-ester/day was added. While 51 patients commenced the study, one patient developed a skin rash after 2 days of EPA, and thus 50 patients were evaluated. An estimation of study size was not reported. AF was assessed by self-recorded electrocardiograms. The primary endpoint, AF burden, defined as the number of days of AF per month, was not different before and after the intervention with EPA (Watanabe et al., 2011).

The systematic review already mentioned additionally included other trials on recurrent AF published in abstract form, and found an overall reduction of recurrent AF of 17% (OR, 95%CO 0.48–1.45; Liu et al., 2011). Taken together, epidemiologic findings and the results of the intervention trials reported thus far make prevention of recurrent AF after electric cardioversion with EPA + DHA a promising target for further research. A number of such studies are currently ongoing (c.f. clinicaltrials.gov).

VENTRICULAR ARRHYTHMIAS

Intermediate parameters

In an epidemiologic study in 260 patients with acute myocardial infarction, intake of omega-3 fatty acids was found to correlate inversely with premature ventricular beats (Smith et al., 2009). In an epidemiologic study in 707 Alaskan natives, a significant negative association between heart rate and the omega-3 Index was seen (Ebbesson et al., 2010). In two small studies, VT's were found to be less inducible in the electrophysiology laboratory after a 6-week intake of 900 mg EPA + DHA/day, or acute infusion of 3.9 g EPA + DHA (Metcalf et al., 2008; Madsen et al., 2010).

Studies in carriers of an implanted cardioverter-defibrillator

A meta-analysis on the three trials evaluating the effects of EPA and DHA in a total of 1148 carriers of an implanted cardioverter-defibrillator (ICD) concluded that "These findings do not support a protective effect of omega-3 PUFAs from fish oil on cardiac arrhythmia in all patients with an ICD," and felt the same to be true for all subgroups (Brouwer et al., 2010). A retrospective analysis of the 566 participants carrying an ICD in the GISSI-Heart failure trial found a similar result, but concluded that "The results of this study, though not statistically significant, support prior evidences of an anti-arrhythmic effect of $n-3$ PUFA in patients with ICD" (Finzi et al., 2011). Of note, a relatively small study in 102 German carriers of an ICD found that the higher the content of red blood cells in EPA + DHA, the higher the likelihood of an action of the ICD (Wilhelm et al., 2008). Interestingly, the authors noted that "These arrhythmias (VT's) were more often monomorphic and had a longer average cycle length, suggesting reentrant mechanism. These arrhythmias are not necessarily life-threatening." In a randomized controlled trial in 44 carriers of an ICD, among other parameters, microvolt T-wave analysis improved, heart rate variability increased and the heart rate of non-sustained VT decreased (Nodari et al., 2009) Taken together, the results of the ICD-studies support the need to differentiate the cause of the action of the ICD (VT vs. VF), and the action of the ICD (anti-tachycardia pacing vs. shock) in such studies, as discussed earlier (von Schacky, 2008) to get a clearer picture of the effects of EPA and DHA in carriers of an ICD.

Sudden cardiac death

In an epidemiologic study, long-term fish consumption was associated with a lower risk for SCD (Streppel et al., 2008). This is in keeping with the observation that a low omega-3 index increased the risk of ventricular fibrillation during the acute ischemic phase of a myocardial infarction (Aarsetoey et al., 2008), and the observation that a low omega-3 index was found to be associated with SCD (Aarsetoey et al., 2011). Of note, a 1% increase of the omega-3

index was associated with a 58% (95% CI 0.25–0.76%) reduction in risk of VF during the acute phase of a myocardial infarction (Aarsetoey et al., 2011).

Intervention trials with clinical endpoints

Late 2008, a systematic review of randomized controlled trials of fish oil as dietary supplements in humans found a non-significant reduction in SCD (odds ratio, OR0.81, 95% CI 0.52–1.25), but a significant reduction in death from cardiac causes (OR0.80, 95% CI 0.69–0.92; León et al., 2008). SCD is an elusive endpoint, since it occurs only in 1.4% of a population after a myocardial infarction (Marchioli et al., 2002), mandating large study sizes or meta-analyses of large studies for any intervention to demonstrate an effect. For that reason, SCD is rarely selected as a primary endpoint of an intervention study. Combining important clinical events is the preferred approach.

An example was the Heart Failure trial by the Italian GISSI-group (Gissi-HF Investigators et al., 2008) Patients with chronic heart failure of New York Heart Association class II–IV were recruited, and randomly assigned to 850 mg EPA + DHA as ethyl-ester daily ($n = 3494$) or to a matching placebo ($n = 3481$). The trial had two co-primary endpoints: time to death and time to death or admission to hospital for cardiovascular reasons. Study size was estimated based on a 15% relative reduction of the expected absolute mortality rate of 25% during 3 years and 90% power and a two-sided significance of 0.045. Analysis was by intention-to-treat. EPA + DHA were as safe and as tolerable as placebo. After 3.9 years, time to death was reduced by 9% [adjusted hazard ratio (HR) 0.91, 95.5% CI 0.833–0.998, $p = 0.041$], while time to death or admission to hospital for cardiovascular reasons was reduced by 8% (adjusted HR 0.92, 99% CI 0.849.0.999, $p = 0.009$). Adjustments were predefined and due to baseline inequalities. In the intervention group, 274 participants (7.8%) died a presumably arrhythmic death, in contrast to 304 participants (8.7%) in the placebo group (adjusted HR 0·88, 95% CI 0·75–1·04, $p = 0.141$). SCD occurred in 307 (9%) patients allocated to $n-3$ PUFA and 325 (9%) in the placebo group (adjusted HR 0.93, 95% CI 0.79–1.08, $p = 0.333$). The authors concluded that "$n-3$ PUFA can provide a small beneficial advantage in terms of mortality and admission to hospital for cardiovascular reasons in patients with heart failure" (Gissi-HF Investigators et al., 2008).

The Alpha-Omega Trial was a multi-center, double-blind, placebo-controlled trial in 4837 patients after a myocardial infarction (Geleijnse et al., 2010; Kromhout et al., 2010). Patients were randomly assigned to receive four trial margarines: a margarine supplemented with 400 mg of EPA + DHA, a margarine supplemented with 2 g of ALA, a margarine supplemented with both, or a placebo margarine. The primary end point was a combination of fatal and non-fatal cardiovascular events and cardiac interventions, but SCD was not specifically followed. Study size estimation was initially based on a CHD mortality of 4% per year, and the usual assumptions, but had to be adjusted because of a lower mortality observed. Adjustment led to the combined endpoint just mentioned. Analysis was by intention-o-treat. Adverse events were evenly distributed among the four groups. None of the interventions reduced the primary endpoint. The authors concluded that "Low-dose supplementation with EPA–DHA or ALA did not significantly reduce the rate of major cardiovascular events among patients who had had a myocardial infarction." Reasons for the neutral result discussed by the authors were specifics of the study population, concomitant pharmacologic treatment, and the low-dose given (Kromhout et al., 2010). Elsewhere, a relatively high background intake of EPA + DHA was discussed (Mozaffarian and Wu, 2011). More recently, the authors of the Alpha-Omega Trial reported a protective effect against ventricular arrhythmia-related events in the subgroup of post-MI patients with diabetes (Kromhout et al., 2011).

At least in the field of omega-3 fatty acids, the only trial ambitiously using SCD as a primary endpoint was "OMEGA," a randomized, placebo-controlled, double-blind, multi-center trial comparing 1 g/day EPA + DHA as an ethyl-ester to an olive oil placebo for 1 year in 3851 German patients shortly after a myocardial infarction (Rauch et al., 2010). Study size was estimated based on previous registry data with SCD projected to occur in 1.9% in the group treated with omega-3 acid ethyl-esters 90 and 3.5% in the placebo group, and alpha of 2.5% and a power of 80% (Rauch et al., 2006). Primary analysis was by intention-to-treat, and SCD occurred in 1.5% of both verum and placebo groups, with other cardiovascular events also evenly distributed. As is, OMEGA had a statistical power of 44% to detect the assumed reduction in SCD. This power obviates any conclusion on the effect of EPA + DHA, because it remains unclear, whether the intervention tested was ineffective or the study size was too small. The authors discussed an unexpected low rate of SCD, cross-contamination by in increase in fish consumption, and improvements in drug therapy as reasons for their results. The authors concluded that "Guideline-adjusted treatment of acute myocardial infarction results in a low rate of SCD and other clinical events within 1 year of follow-up, which could not be shown to be further reduced by the application of omega-3 fatty acids." As mentioned, the power of the study does not permit conclusions with regard to the therapeutic effect of EPA + DHA. Moreover, in GISSI-P, in the first year after a myocardial infarction, the incidence of SCD in the control population was 1.4%, basically identical to the incidence observed in OMEGA (Marchioli et al., 2002). In GISSI-P, a highly comparable study population participated, and treatment conforming current guidelines was less frequently applied. Therefore, the conclusion of the authors of OMEGA is not only scientifically unsound, but also not supported by the data presented. Strangely enough, the accompanying editorial reiterated the conclusions of OMEGA (Eckel, 2010).

The French SuFOL.OM3 trial was a randomized double-blind multi-center trial, and recruited 2501 participants with a history of myocardial infarction, unstable angina, or ischemic stroke (Galan et al., 2010). In a factorial design, 600 mg EPA + DHA ethyl-ester was compared to vitamins, a combination of the two, or placebo. The primary endpoint was the first major cardiovascular event (e.g., non-fatal and fatal myocardial infarction, stroke, SCD, and others). Study size was based on an annual event rate of 0.087, a 10% relative reduction by one intervention (19% by two), a power of 90%, and a two-tailed α of 0.05. In the 4.7-year follow-up period, an event occurred in 6.3% of participants, with no statistically significant differences between groups. The authors discussed

low-dose and low statistical power due to a lower than expected event rate as explanations for the neutral results. The authors concluded that "the study does not support the routine use of dietary supplements containing B vitamins or omega-3 fatty acids for prevention of cardiovascular disease" "after the acute phase of the initial event." Again, the power of the study does not permit conclusions with regard to the therapeutic effect of EPA + DHA, because of lack of sufficient statistical power.

THE OMEGA-3 INDEX PERSPECTIVE

The omega-3 index was defined as the percentage of EPA + DHA in red cells, as determined with a highly standardized analytical method, currently installed in a few laboratories around the world (Harris and von Schacky, 2004). While mean levels of the omega-3 index differ from population to population, mean levels had a statistically normal distribution in all populations studied so far (von Schacky, 2011). Therefore, in any population, some individuals have relatively high, and some have relatively low levels, while most are in between. The omega-3 index reflects tissue levels of EPA + DHA, e.g., of the heart (von Schacky, 2011). A low omega-3 index is a strong predictor of future adverse cardiovascular events (von Schacky, 2011).

Using the omega-3 index to answer the question whether EPA + DHA are anti- or pro-arrhythmic provides a clearer picture than the one provided thus far. In Japan, the mean omega-3 index was found to be 9.58% in unselected persons, and the incidence of SCD in the general population 7.8/100,000 person years, while in Western countries, the mean omega-3 index is frequently among 5%, and the incidence of SCD in the general population is 150/100,000 person years (von Schacky, 2010). In two case–control studies on SCD in the US, levels of omega-3 fatty acids in red cells or whole blood were inversely related to risk for SCD, and a 10-fold difference in risk was reported (von Schacky, 2010). Thus, a high omega-3 Index, e.g., 8–11%, is associated with a low-risk for SCD, and risk appears to increase with decreasing values of the omega-3 index.

Experimental evidence indicates that acute increases in EPA + DHA concentrations do not have a further anti-arrhythmic effect in tissues already replete with them (Den Ruijter et al., 2010). Therefore, it is unlikely that an anti-arrhythmic effect can be demonstrated in persons or populations with a high omega-3 index, like in Japan. In keeping, in a large randomized intervention study in persons at elevated risk for cardiovascular disease in Japan (20% of whom had established cardiovascular disease), 1.8 g EPA/day did not reduce SCD (Yokoyama et al., 2007). Of note, the incidence of SCD in JELIS was 40/100,000, substantially lower than in the general population in Western countries, as mentioned above (von Schacky, 2010). However, acute increases in EPA + DHA, e.g., by infusion or after a 6-week dietary intervention reduced inducibility of ventricular tachycardias, but only in subjects with low levels (Schrepf et al., 2004; Metcalf et al., 2008; Madsen et al., 2010). Although the latter studies were small and short, current evidence indicates that acute increases of EPA + DHA levels by infusion or a short-term dietary intervention can reduce the probability of ventricular arrhythmias in those with suboptimal levels, but that high tissue levels, reflected by a high omega-3 index, are protective.

A lower omega-3 index also makes other adverse outcomes more likely than a higher omega-3 index: Among the outcomes are ventricular fibrillation during a myocardial infarction (Aarsetoey et al., 2011), myocardial infarctions (Block et al., 2008; Park et al., 2009), mortality after a myocardial infarction (Pottala et al., 2010), and many more reviewed elsewhere (von Schacky, 2011). Supporting evidence is provided by plasma measurements of EPA + DHA (e.g., Mozaffarian et al., 2011a).

The omega-3 index has a lower biological variability than plasma or whole blood EPA + DHA (Harris and Thomas, 2010). The level of the omega-3 index is determined by many factors, among them catabolism of EPA + DHA, age, body mass index, and intake of EPA + DHA (von Schacky, 2011). Intake, however, explains <16 or 12% of the variability of EPA + DHA in the red cell membrane (Ebbesson et al., 2010; Sala-Vila et al., 2011). This may partly explain, why epidemiologic studies focusing on intake of EPA + DHA provided less clear results than epidemiologic studies focusing on the omega-3 index (von Schacky, 2010). In the future, a more widespread use of the omega-3 index in research projects will provide a clearer picture of associations between EPA + DHA and cardiovascular events. Already now, however, the omega-3 index fulfills important criteria of the US Preventive Services Task Force or the American Heart Association for new biomarkers for cardiovascular risk (Helfand et al., 2009; Hlatky et al., 2009):

1. Ease and reliability of measurement. The analytical methodology is standardized in three laboratories around the world, and conforms to the rules of Clinical Chemistry (e.g., plausibility checks, proficiency testing).
2. Risk predicted independent of conventional risk factors. This has been demonstrated for an American and a Korean population, and was evidenced by a larger area under the curve in comparison to the Framingham index (Park et al., 2009; Shearer et al., 2009).
3. Reclassification of persons at intermediate risk (Shearer et al., 2009).
4. Therapeutic consequence. In all populations studied so far, an increase in intake of EPA + DHA increased the mean omega-3 index. In meta-analyses, increased intake of EPA + DHA resulted in decreased occurrence of major cardiovascular events (e.g., León et al., 2008; Mozaffarian and Wu, 2011). Whether an omega-3 index based supplementation with EPA + DHA is superior to the current untargeted use of EPA + DHA remains to be formally demonstrated, however.

DISCUSSION

Only recently, bioavailability problems of preparations of EPA + DHA have surfaced. Unfortunately, while ethyl-esters have been used in most intervention trials, ethyl-esters are the least well absorbed chemical form of omega-3 fatty acids, and absorption depends on the fat content of the meal eaten with intake (Dyerberg et al., 2010; Neubronner et al., 2011). A lower bioavailability translates into a lower biological activity (Schuchardt et al., 2011). Moreover, large differences exist in absorption of EPA + DHA from individual to individual, even if an identical dose of a chemical form is taken in an identical matrix, in this case 940 mg EPA + DHA from salmon oil (triglyceride) in 200 ml of a drink

(Köhler et al., 2010). Both phenomena impact negatively on the differentiation between intervention and control groups in terms of dose of EPA + DHA absorbed.

Moreover, in all intervention trials mentioned, participants were recruited irrespective of their baseline levels of EPA + DHA. Participants with a high omega-3 index at baseline are not likely to develop a cardiovascular event during a trial. In addition, levels of EPA + DHA achieved in intervention and control groups during the study will overlap in a substantial portion of study participants, again negatively impacting on the differentiation between intervention and control groups in terms of levels of EPA + DHA achieved. Jointly, the two effects just mentioned produce a tendency toward neutrals results of clinical trials with EPA + DHA. In the future, recruiting study participants with a low baseline omega-3 index, and treating to a target omega-3 index (e.g., 8–11%) will make intervention trials with EPA + DHA more efficient. Very efficient trials are needed to demonstrate effects of any intervention on rare endpoints, like SCD or new-onset AF.

Pro-arrhythmic effects have not been seen in any intervention trial with clinical endpoints, rather arrhythmic events were either reduced or not significantly altered (Marchioli et al., 2002; Gissi-HF Investigators et al., 2008; León et al., 2008; Galan et al., 2010; Kromhout et al., 2010; Rauch et al., 2010; Mozaffarian and Wu, 2011). The same phenomenon has been seen in the animal studies reviewed above. This is in keeping with all but one epidemiologic study reporting slower VT's the higher the red cell content of EPA + DHA (Wilhelm et al., 2008). Thus, the data from epidemiologic and intervention studies are compatible with the speculation that EPA + DHA inhibit degeneration of non-fatal VT into fatal VF. A re-analysis of the original data obtained in the three intervention trials in carriers of an ICD would be a first step toward substantiating this speculation.

Currently, it is unclear, whether the anti-arrhythmic effect of EPA + DHA is restricted to arrhythmias associated with ischemia or not. The data reported from the large clinical trials do not answer this question. The three small studies on inducibility of VT's in the electrophysiology laboratory argue in favor of an effect in the absence of ischemia (Schrepf et al., 2004; Metcalf et al., 2008; Madsen et al., 2010), as do results from studies using intermediate or surrogate parameters, such as heart rate, heart rate variability, or rate of VT's (discussed above). The observation from Norway that an 1% increase in the omega-3 index was associated with a 58% reduction in risk of VF during the acute phase of a myocardial infarction (Aarsetoey et al., 2011), supports the notion that the anti-arrhythmic effect of EPA + DHA becomes specifically important in the presence of ischemia. Based on current evidence, this question cannot be resolved with certainty, however (Raitt, 2009).

CONCLUSION

Taken together, there is no evidence that EPA + DHA are arrhythmic at the atrial level, i.e., that EPA + DHA propagate AF. Rather, intervention trials so far indicate that there might be a small preventive effect that needs to be investigated in larger and/or more efficient trials than conducted so far. This is true for postoperative AF, recurrent AF and new-onset AF. On the ventricular level, epidemiologic studies indicate a possible anti-arrhythmic effect, possibly by slowing VT, thus possibly preventing progression to VF. Results of trials on intermediate endpoints in carriers of an ICD were inconclusive, possibly because of indiscriminate combination of endpoints. SCD is a rare event. Unfortunately, the only intervention trial to study SCD as a primary endpoint was inadequately powered, obviating a conclusion on the preventive effect of EPA + DHA on SCD. Other trials used combinations of endpoints, usually including an arrhythmic endpoint and in no trial, a pro-arrhythmic effect was found. Due to problems with study power in another trial, and the possibility of inadequate dosing in yet another, the anti-arrhythmic effect of EPA + DHA was recently demonstrated in only one trial in patients with congestive heart failure. All trials thus far suffer from a tendency toward a neutral result, since study participants were recruited irrespective of their baseline omega-3 index. Rather than indiscriminately recruiting study participants, use of a low omega-3 index as an inclusion criterion will make more efficient intervention trials possible, and will lead to a clearer delineation of mechanisms of action. Use of the omega-3 index provides a more targeted approach toward defining the impact of EPA + DHA and/or their derivatives on cardiac rhythm.

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Do omega-3 fatty acids have a role in prevention of cardiovascular disease?

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As often the case within scientific research, the answer is not as straightforward as the question. In contradistinction to earlier data, recently published studies have been negative, and thus raised the question of whether supplementation with omega-3 fatty acids for prevention of cardiovascular disease is now passé. Some background knowledge is necessary to appreciate this perplexing and controversial topic.

Observational studies performed within the general population over the past 40 years have almost uniformly noted an inverse relationship between fatty fish or *n*–3 fatty acid consumption and morbidity or mortality from coronary heart disease (CHD; Kromhout et al., 1985, 2010, 2011; Dolecek, 1992; Rodriguez et al., 1996; Daviglus et al., 1997; Albert et al., 1998, 2002; Oomen et al., 2000; Iso et al., 2001; Yuan et al., 2001; Hu et al., 2002, 2003; Lemaitre et al., 2003; Mozaffarian et al., 2003; He et al., 2004; Panagiotakos et al., 2005; Mozaffarian and Rimm, 2006; Bjerregaard et al., 2010). Likewise, among studies which measured blood or tissue levels of *n*–3 fatty acids the majority have shown the same inverse correlation with cardiovascular disease events (Siscovick et al., 1995; Albert et al., 1998, 2002; Harris et al., 2007; Block et al., 2008; Park et al., 2009; Pottala et al., 2010). However, observational data can never prove cause-and-effect. Randomized clinical trials (RCT) have been designed specifically to provide a more controlled evaluation of the effects of omega-3 fatty acids treatment on adverse cardiovascular events. However, RCTs have their own limitations, especially when the interventional agent being tested is one that is available in food and consumed in varying amounts by the population participating in the trial. Among the vitamin supplement trials, for example, results derived from the whole study population have sometimes been opposite from the results from the "deficient" subpopulation (Morris and Tangney, 2011; Rimm and Stampfer, 2011).

There have been about 20 published trials in which patients were randomized to a daily dose of *n*–3 fatty acid vs. placebo and who were then followed for varying intervals in order to assess some type of cardiovascular disease outcome. Most of these were performed in patients with a history of CHD.

Two recently published meta-analyses reached divergent conclusions. Kwak et al. (2012) performed a meta-analysis of 14 trials, and concluded that there was insufficient evidence of a preventive effect of omega-3 fatty acid supplements against overall cardiovascular events among patients with a history of cardiovascular disease. The authors note that there was a small reduction in cardiovascular death (RR 0.91; 95% CI: 0.84–0.99), which disappeared when one study with major methodological problems was excluded. Quality of a meta-analysis in predicated upon the quality of the individual studies and on the amount of heterogeneity among the studies. In this case, all but four of these trials had less than 600 participants, half lasted no more than a year, several were designed with angiographic endpoints, and three were performed in patients with implantable cardioverter-defibrillators (ICDs), using ICD discharges as the primary endpoint. General conclusions from such a meta-analysis are therefore highly speculative. The Agency for Healthcare Research and Quality also performed a systematic review with random effects meta-analysis (EPC Technical Papers Series, 2012). They included RCTs of at least 4 weeks duration, using EPA + DHA supplementation less than 6 g/day. The summary relative risks for all-cause mortality (17 trials, 51,264 patients) and cardiovascular mortality (14 trials, 48,500 patients) were 0.95 (95% CI: 0.89–1.01) and 0.89 (95% CI: 0.83–0.96), respectively. Whether you believe omega-3 fatty acid supplementation reduces cardiovascular mortality or not would seem to depend on which meta-analysis you prefer. However, since the studies included in both of these reviews are so diverse, a meta-analysis is not the most appropriate method to answer our question. A look at the differences among the large RCTs which were specifically designed to assess cardiovascular morbidity and mortality will shed more light, and at least clarify what are the unresolved issues.

Only eight RCTs have been of sufficient size ($n = 2000–18,000$) to provide adequate power for detecting statistically meaningful results (Kromhout et al., 1985, 2010, 2011; Burr et al., 1989; GISSI-Prevenzione Investigators, 1999; Yokoyama et al., 2007a,b; The GISSI-HF investigators, 2008; Galan et al., 2010; Rauch et al., 2010; The ORIGIN Trial Investigators, 2012). Trial designs, *n*–3 fatty acid doses, and study population characteristics were quite different. In summary, GISSI-Prevenzione Investigators (1999) and DART (1989) showed a large CV mortality benefit; JELIS (2007) showed a reduction in non-fatal CV events, but no effect on mortality; The GISSI-HF investigators (2008) showed a small mortality benefit in CHF patients; The ORIGIN Trial Investigators (2012), Omega (2010), Alpha-Omega (2010), and SU.FOL.OM3 (2010) showed no CV benefits (fatal or non-fatal) in their overall trial results. It should also

be noted that there are a few clinical and experiment studies in which omega-3 fatty acids adversely altered cardiac rhythm, but effects of omega-3 fatty acids on rhythm disturbances is a separate, albeit related, topic which will not be addressed here.

The most popular explanation for the divergent outcomes is the following: The trials showing the largest benefit were older, performed in the pre-statin era, which was also a time of fewer therapeutic options in general for the patient presenting with a CVD event (i.e., virtually no revascularizations in the acute coronary syndrome setting, less anti-platelet therapies, etc). Patients participating in the more recent trials had the advantage of much more aggressive interventions as well as drug therapies that have been proven to reduce recurrent event rates, especially the statin class. Therefore, the mechanism(s) by which omega-3 fatty acids formerly improved CV outcomes may simply have been obviated by the newer better therapies.

However, the divergent outcomes of the omega-3 studies published to date also raise the possibility that only certain subgroups of patients derive a cardiovascular benefit from taking omega-3 fatty acids. Factors which may be critical in identifying the most responsive subgroups, include the following:

1. Cardiac function – In GISSI-P there was an inverse association between ejection fraction (EF) and prevention of sudden death. GISSI-HF (mean EF 33%) showed a 9% reduction in total mortality for HF patients (14% reduction in those who were compliant with medication). Patients with mildly diminished cardiac function (EF 30–45%), a group which were not systematically studied in the three negative post-MI trials (Omega, Alpha-Omega, and SU.FOL.OM3) and the one negative trial of dysglycemia patients (The ORIGIN Trial Investigators, 2012), might still be appropriate candidates for omega-3 therapy.
2. Baseline omega-3 intake – In contrast to the results from the ORIGIN trial (all participants had diabetes or pre-diabetes), the Alpha-Omega sub-study of its 1,014 diabetic post-MI patients showed that the EPA + DHA group, compared to the placebo group, had a hazard ratio (HR) of 0.51 for death from CHD (p = 0.04), and 0.51 for ventricular arrhythmia-related events (p = 0.09). In addition, the EPA + DHA + ALA group had a HR of 0.16 for ventricular arrhythmia-related events, and a HR of 0.28 for ventricular arrhythmia-related events + fatal MI (Kromhout et al., 1985, 2010, 2011). Observational data have shown not only that EPA + DHA intake up to 200–250 mg/day is associated with decreased cardiac, cardiovascular, or sudden cardiac death, but that no further reduction in fatal CHD occurs when EPA + DHA intakes exceed 200–250 mg/day (Mozaffarian and Rimm, 2006; EPC Technical Papers Series, 2012). Median baseline intake in ORIGIN was 210 mg/day, in Alpha-Omega it was 120–130 mg/day. A high-baseline intake of omega-3 fatty acids is the reason various authors have proposed for why there was no CV mortality benefit observed in the JELIS trial, whose Japanese participants had baseline blood levels of EPA + DHA roughly 10 times higher than the average American level (Mozaffarian, 2007; Yokoyama et al., 2007a,b). It also explains why the control group in JELIS had a cardiac death rate per 1000 person-years of 2.5, while in GISSI-Prevenzione it was 17. It's likely that the baseline omega-3 intake (or more critically the tissue level, for which the estimated intake is a rough correlate) is another important factor in determining which patients will derive a benefit in prevention of (specifically) fatal CHD.
3. Baseline triglyceride (TG) levels – ORIGIN patients had a median TG level of 140 mg/dL. The Alpha-Omega subgroup of diabetic patients, in whom omega-3 fatty acid therapy reduced CHD death by ~50%, had a mean baseline value of 198 mg/dL. The JELIS subgroup of diabetic/pre-diabetic patients, who had a 22% reduced incidence in CAD events in the omega-3 arm, had a mean TG value of 175 mg/dL (Oikawa et al., 2009). The overall JELIS trial showed an inverse relationship between TG levels and benefit in CV event reduction, with no benefit in the subgroup with baseline TG levels <150 mg/dL and HDL-C >40 mg/dL. High TG and/or low HDL might possibly be a marker for who derives some CVD benefit from omega-3 supplementation.
4. Omega-3 dose – All of the large trials to date have used doses of 1 g or less per day, except JELIS, which although an open-label study, showed a 19% reduction in non-fatal CV events. Therefore, what is the most effective dose remains an unanswered question that will not be resolved by any of the studies currently in progress (ASCEND, Rishio e Prevenzione, and VITAL, all of which use <1 g/day).

At this time, it is not possible to definitively answer our original question. The factors enumerated above may or may not explain the discrepancies observed in the omega-3 trial outcomes, but each one formulates a reasonable hypothesis which needs to be addressed in future research. Otherwise, those who believe the latest research has now established that there is no role for omega-3 fatty acids in prevention of CVD may be throwing the proverbial baby out with the bathwater, and thereby deprive certain patients a potentially valuable therapy.

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Why do we still need large scale clinical trial: the case of *n*−3 PUFA

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After the first reports about a protective effect on coronary heart disease (CHD) published more than 40 years ago, wide interest in the therapeutic use of *n*−3 polyunsaturated fatty acids (*n*−3 PUFA) aroused. Since then, many studies and meta-analyses have reported a significantly reduced risk of CHD and CV death due to fish and *n*−3 PUFA intake. Some of the overviews reported a significant reduction of risk of sudden cardiac death, all-cause death, and nonfatal CV events. On the other side, recent clinical trials had mixed findings, raising concern about the consistency of the evidence on *n*−3 PUFA. We critically reviewed recent large clinical trials reporting data on the antiarrhythmic effects of *n*−3 PUFA in different clinical settings, i.e., patients with CHD, heart failure, with implantable cardioverter defibrillator, and at risk of atrial fibrillation, in order to summarize the results which are available up to date and possibly give "substantiated" fuel to the debate on the conflicting results of *n*−3 PUFA.

Keywords: *n*−3 polyunsaturated fatty acids, clinical trials, review

INTRODUCTION

After the first report about a protective effect on coronary heart disease (CHD) published more than 40 years ago, wide interest in the therapeutic use of *n*−3 polyunsaturated fatty acids (*n*−3 PUFA) aroused (Bang et al., 1971; Bang and Dyerberg, 1972). Since then, many studies have been performed, increasing our knowledge on the cardiovascular (CV) effects of *n*−3 PUFA. Various meta-analyses have reported a significantly reduced risk of CV events, primarily due to reduction of CHD and CV death due to fish and *n*−3 PUFA intake (He et al., 2004; Leon et al., 2008; Marik and Varon, 2009; Mente et al., 2009). Some of the overviews reported a significant reduction of risk of sudden cardiac death (SCD; Leon et al., 2008; Marik and Varon, 2009), all-cause death (Marik and Varon, 2009), and non-fatal CV events (Marik and Varon, 2009). However, several questions remain open to date, e.g., the mechanisms accounting for the benefit observed in clinical studies as well as a possible heterogeneity of the effect in different populations. On the other side, recent clinical trials had mixed findings, raising concern about the consistency of the evidence on *n*−3 PUFA.

Among the many physiological effects which *n*−3 PUFA are supposed to have, the antiarrhythmic effect is the most interesting one but is challenging to be documented in humans because of the absence of reliable physiological measures or biomarkers to quantify the antiarrhythmic potential of *n*−3 PUFA on SCD.

We critically reviewed recent large clinical trials reporting data on the antiarrhythmic effects of *n*−3 PUFA in different clinical settings, i.e., patients with CHD (Marchioli et al., 2002; Yokoyama et al., 2007; Rauch et al., 2010), heart failure (HF; Tavazzi et al., 2008), with implantable cardioverter defibrillator (ICD; Leaf et al., 2005; Raitt et al., 2005; Brouwer et al., 2006), and at risk of atrial fibrillation (AF; Calo et al., 2005; Kowey et al., 2010; Nodari et al., 2011), in order to summarize the results which are available up to date and possibly give "substantiated" fuel to the debate on the conflicting results of *n*−3 PUFA.

CORONARY HEART DISEASE

The first intervention study suggesting an antiarrhythmic effect of *n*−3 PUFA was the Diet and Reinfarction Trial (DART) study carried out on 1989 (Burr et al., 1989). In 2033 post-myocardial infarction (MI) men, those advised to eat fat fish two to three times a week had a significant 29% reduction in fatal MI compared to those not advised so. This benefit appeared early after the start of the trial and was hypothesized to be due to a reduction of sudden death. Some years later, a decreased mortality and sudden death were observed in post-MI patients receiving a Mediterranean alpha-linolenic acid (ALA)-rich diet (de Lorgeril et al., 1994). In another trial, patients with suspected MI and receiving fish oil (2 g *n*−3 PUFA daily) or mustard seed oil (containing 2.9 g of ALA per day) experienced fewer sudden deaths than the placebo group (Singh et al., 1997). An analysis of the US Physician's Health Study, a prospective study on 20,551 male physician followed for up to 11 years, showed that fish consumption ≥ once per week was associated with a 52% reduction in the risk of SCD as compared with men with lower fish intake (Albert et al., 1998). Later, a new report from this study showed an inverse relationship between blood levels of long chain *n*−3 PUFA and risk of SCD (Albert et al., 2002).

The strongest evidence suggesting an antiarrhythmic effect of *n*−3 PUFA was provided by GISSI-Prevenzione, an open-label, randomized, controlled trial performed to test the efficacy of an oral administration of *n*−3 PUFA (1 g daily) and vitamin E on

morbidity and mortality in 11,323 Italian patients with recent (MI < 3 months; Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico, 1999). After 3.5 years of follow-up, $n-3$ PUFA therapy significantly reduced the first combined, primary endpoint (death, non-fatal myocardial, and non-fatal stroke) by 15% (95% confidence interval, CI: 3–27, $P = 0.02$) as well as the co-primary endpoint (cardiovascular death plus non-fatal MI plus non-fatal stroke) by 20% (95% CI: 6–32, $P = 0.006$) as compared to the control group. Secondary analyses of the components of the primary endpoints showed that almost all the benefit observed in the combined endpoints was attributable to the reduction in fatal events: total mortality 20%, (95% CI: 6–23%); CV death 30% (95% CI: 13–44%); CHD death 35% (95% CI: 16–49%); and SCD 44% (95% CI: 24–60%). An intention-to-treat analysis of GISSI-Prevenzione, adjusted for interaction between treatments, was aimed at assessing the time course of benefit on fatal events. It showed an early divergence of the survival curves for $n-3$ PUFAs and control groups after randomization, with total mortality being significantly lowered after only 3 months of treatment (RR 0.59, 95% CI: 0.36–0.97; $P = 0.037$; Marchioli et al., 2002). Likewise, the reduction in risk of SCD was already statistically significant at 4 months (RR 0.47; 95% CI: 0.219–0.995; $P = 0.048$). A similarly significant, although delayed, pattern was observed for CV, cardiac, and CHD deaths after 6–8 months of treatment. In agreement with previous evidence coming from both experimental and human studies, such findings suggested that the early benefits of $n-3$ PUFA may be due to their antiarrhythmic activity (McLennan et al., 1992, 1993; Kang and Leaf, 1994, 1995, 1996; Kang et al., 1995; Sellmayer et al., 1995; Siscovick et al., 1995; Xiao et al., 1995, 1997, 1998; Christensen et al., 1997; Billman et al., 1999).

GISSI-Prevenzione trial had several strengths. It was adequately sized (the largest prospective controlled trial that investigated the effect of a dietary-derived drug on CV events) in a population of patients at high-risk of arrhythmia, with a reliable statistical power to show that $n-3$ PUFA could decrease hard endpoints of morbidity and mortality in a clinically and statistically significant manner. As to clinical relevance, it is worth noting that for every 1000 post-MI patients treated for 1 year with 1 g daily of $n-3$ PUFA, 5.7 lives were saved. Such protective effect with $n-3$ PUFA treatment observed in GISSI-Prevenzione is comparable in the magnitude to the results of other drugs which are now considered therapeutic cornerstone in cardiovascular prevention. For instance, in the LIPID trial, 5.2 lives could be saved treating with pravastatin for 1 year 1000 patients with hypercholesterolemia and CHD (The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group, 1998). Although some changes in the management of post-MI patients occurring over years have to be considered, many aspects of GISSI-Prevenzione are reassuring as to the transferability of the results to clinical practice for post-MI patients,: (a) the trial was very large and performed in the framework of a "usual care" clinical setting, (b) it was carried out in a country-wide network of hospitals within the Italian national public health service, and (c) had a pragmatic design aimed at not interfering with daily clinical practice. It might be also argued that in populations at high CV risk, such as those with a western lifestyle, $n-3$ PUFA might have greater benefit than in the Italian population, known to be at low risk for CHD mainly for the Mediterranean dietary habits (Marckmann and Gronbaek, 1999). The benefit of $n-3$ PUFA treatment can be considered as additive to those of recommended pharmacological treatments and lifestyle interventions. Indeed, patients enrolled in GISSI-Prevenzione received $n-3$ PUFA treatment on the top of preventive pharmacological treatments, including aspirin, beta-blockers, angiotensin-converting enzyme (ACE) inhibitors and cholesterol-lowering drugs, as well as of lifestyle interventions leading at the end of the trial to a positive modification of dietary habits with increase of the (already relatively high) intake of olive oil, fruit, vegetables, and fish (Barzi et al., 2003).

Recent randomized clinical trials did not confirm the antiarrhythmic effect of $n-3$ PUFA. The Japan EPA Lipid Intervention Study (JELIS; Yokoyama et al., 2007) was a large scale trial conducted in 18,000 hypercholesterolemic Japanese patients who were randomized to $n-3$ PUFA treatment (1.8 g/day) in combination with statin vs. statin alone. After a mean follow-up of 4.6 years, the patients in the $n-3$ PUFA group experienced a reduction of 19% of CHD events. The benefit of $n-3$ PUFA was limited to non-fatal CHD events, particularly unstable angina, whilst the effect on fatal events was not significant, particularly on SCD. Such apparent lack of the antiarrhythmic effect in this population of patients could be related to some specific aspects of JELIS. Firstly, the assumption of reaching a statistical power of 80% for detecting a relative reduction of 25% in the primary endpoint was respected, but the rate of SCD (i.e., a secondary outcome measure) in the JELIS population was extremely low. Only 35 of the 18,645 participants (0.19%) in JELIS experienced a SCD during 5 years of follow-up. As a result the study lacked sufficient power, to detect an effect on mortality in general and specifically on SCD (statistical power of 13, 7, and 5% to detect a relative reduction of 30, 20, and 10% of the risk in SCD, respectively, **Table 1**). Another relevant aspect of JELIS was the administration of 1.8 g daily of eicosapentaenoic acid (EPA) on the top of a high dietary intake of $n-3$ PUFA. A pooled analysis of prospective and randomized studies suggested the existence of a dose-response curve for the antiarrhythmic effect of $n-3$ PUFA on SCD, which seems to have a steep slope at modest levels of $n-3$ PUFA intake (<750 mg) and a plateau thereafter, higher doses of $n-3$ PUFA having been hypothesized not having further beneficial effects on SCD (Mozaffarian and Rimm, 2006). Because of the high fish intake (e.g., median 900 mg/day) of Japanese people (Iso et al., 2006), this "threshold" effect might play a role in the JELIS results, e.g., by magnifying anti-atherosclerotic, anti-inflammatory, and plaque stabilizing effects of the high daily supplement of $n-3$ PUFA (1800 mg) while the benefit on arrhythmic events was not detectable due to the low rate of such events in this population. Like in the case of JELIS, the lack of clinical benefit reported in a previous study evaluating the effect of 4 g/day of $n-3$ PUFA, compared to corn oil, in 300 post-MI patients (4–8 days; Nilsen et al., 2001), may be due to both a "threshold effect" as well as to an insufficient study power due to the low event rate observed in the study.

In 2010 the results of the OMEGA trial have been published. The study was a randomized, double-blind, placebo-controlled, multicentre trial, testing the effect of $n-3$ PUFA (1000 mg/day for 12 months) on the rate of SCD in 3851 patients with MI

Table 1 | Recent large clinical trials on the antiarrhythmic effects of n-3 PUFA in patients with CHD.

Study	Patients	Number	Treatment daily dose	Control	Follow-up	Endpoints	Events (%) control goup	RR (95% CI)	Study power for		
									30% RRR	20% RRR	15% RRR
GISSI-P (Marchioli et al., 2002)	Patients with recent (≥3 months) MI	11,323	EPA and DHA (average ratio 1:2) 850–882 mg (alone $n = 2836$; plus Vit.E $n = 2830$)	Vit. E alone ($n = 2830$) or no supplement ($n = 2828$)	3.5 years (38417.9 p/y)	**Death, non-fatal MI and non-fatal stroke**	795 (14.1)	0.85 (0.74–0.98)†	>99	>99	90
						CV death, non-fatal MI and non-fatal stroke	621 (11.0)	0.80(0.68–0.94)‡	>99	97	81
						All fatal events	554 (9.8)	0.79 (0.66–0.93)‡	>99	95	77
						CV deaths	370 (6.5)	0.70 (0.56–0.86)§	>99	83	57
						Cardiac deaths	306 (5.4)	0.65 (0.51–0.82)§	98	75	49
						Coronary deaths	258 (4.6)	0.68 (0.53–0.88)‡	96	67	42
						Sudden deaths	154 (2.7)	0.55 (0.39–0.77)§	80	44	26
						Non-fatal MI	233 (4.1)	0.91 (0.70–1.18)	94	62	38
						Non-fatal stroke	57 (1.0)	1.22 (0.75–1.97)	37	17	11
						Coronary deaths + non-fatal MI	475 (8.4)	0.78 (0.65–0.94)‡	>99	91	69
						fatal + non-fatal stroke	77 (1.4)	1.22 (0.81–1.85)	50	24	14
JELIS (Yokoyama et al., 2007)	Patients with total cholesterol ≥6.5 mmol/L	18,645	EPA 1800 mg/day ($n = 9326$) plus statin	Statin alone ($n = 9319$)	Mean 4.6 years	**Major coronary events**	324 (3.5)	0.81 (0.69–0.95)†	>99	77	51
						Sudden cardiac death	17 (0.2)	1.06 (0.55–2.07)	13	7	5
						Fatal MI	14 (0.2)	0.79 (0.36–1.74)	13	7	5
						Non-fatal MI;	83 (0.9)	0.75 (0.54–1.04)*	55	27	15
						Unstable angina	193 (2.1)	0.76 (0.62–0.95)†	86	54	33
						Revascularization	222 (2.4)	0.86 (0.71–1.05)	93	60	37
						CHD death + MI	113 (1.2)	0.78 (0.59–1.03)*	66	33	19
						Fatal + non-fatal MI	93 (1.0)	0.77 (0.56–1.05)*	57	28	17
						CHD death	31 (0.3)	0.94 (0.57–1.56)	19	9	6
						Non-fatal CHD	297 (3.2)	0.81 (0.68–0.96)†	98	3	47
OMEGA (Rauch et al., 2010)	Patients with recent (3–15 days) MI	3851 (3804 included into the endpoint analysis)	*n*−3 PUFA 1 g/day (EPA 460 mg + DHA 380 mg; $n = 1919$)	Olive oil 1 g/day ($n = 1885$)	1 year	**Sudden death**	29 (1.5)	0.95 (0.56–1.60)	19	10	7
						Total mortality	70 (3.7)	1.25 (0.90–1.72)	47	22	14
						MACCE (total mortality, reinfarction, stroke)	149 (8.8)	1.21 (0.96–1.52)	87	50	30
						Revascularization	482 (29.1)	0.93 (0.80–1.08)	>99	98	86
						ICD-terminated TV/VF	2 (0.1)	4.47 (0.97–20.74)*	<1	<1	<1

(Continued)

Table 1 | Continued

Study	Patients	Number	Treatment daily dose	Control	Follow-up	Endpoints	Events (%) control goup	RR (95% CI)	Study power for		
									30% RRR	20% RRR	15% RRR
ALPHA	Patients aged	4837	EPA-DHA	ALA 2 g/day	Median	**Major cardiovascular**	335 (13.8)	1.01 (0.87–1.17)	>99	82	56
OMEGA	60–80 years		400 mg/day	($n = 1197$) +	40.8 months	**events** (fatal MI;					
(Kromhout	with previous		($n = 1192$) +	placebo	(which	non-fatal MI;					
et al., 2010)	(up to 10 years)		EPA-DHA	($n = 1236$)	included the	revascularization)					
	MI		400 mg/day		first 4–6 weeks	Incident CV disease	185 (7.6)	0.92 (0.75–1.13)	89	53	32
			and ALA		in which all the	CV death	82 (3.4)	0.98 (0.72–1.33)	54	26	16
			2 g/day		patients	Coronary death	71 (2.9)	0.95 (0.68–1.32)	46	22	13
			($n = 1212$)		received	Ventricular	74 (3.0)	0.90 (0.65–1.26)	47	22	14
					placebo	arrhythmia-related					
					margarine)	events					
					15,531 p/y	All-cause death	184 (7.6)	1.01 (0.82–1.24)	89	53	32

*EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ALA, alpha-linolenic acid; vit.E, vitamin E; primary endpoints in bold; RR, relative risk; CI, confidence risk; RRR, relative risk reduction; *$P < 0.10$, †$P < 0.05$, ‡$P < 0.01$, §$P < 0.001$.*

in the previous 3–14 days (Rauch et al., 2006, 2010). Secondary endpoints of OMEGA included total mortality and non-fatal CV events. Also, OMEGA showed no difference in the incidence of SCD between *n*−3 PUFA and placebo groups (1.5 vs. 1.5%; $P = 0.84$). No difference with regard to secondary endpoints was found (total mortality 4.6 vs. 3.7%; $P = 0.18$; major cerebrovascular and CV events 10.4 vs. 8.8%; $P = 0.1$; revascularization in survivors 27.6 vs. 29.1%; $P = 0.34$). Some methodological aspects of the OMEGA trial deserve to be considered. It was assumed that the proportion of SCD during the study should have been 44% of total deaths, therefore resulting in a SCD rate of 3.5% in the control group at the end of the study. The observed incidence of SCD was lower (1.5% in both groups), i.e., less than the half of the expected one, thus making the trial underpowered to detect an antiarrhythmic effect of *n*−3 PUFA (**Table 1**). Another aspect to be considered is the treatment of post-MI patients enrolled in OMEGA Study. Acute revascularization was performed in 81% of patients. Short- and long-term guideline-driven medication was administered in the vast majority of patients: b-blockers, ACE inhibitors/angiotensin receptor blockers (ARB), statins, acetylsalicylic acid, and clopidogrel were prescribed to 94, 91, 94, 95, and 88% of patients, respectively. The large use of guideline-based therapy after MI may have contributed to the low rate of fatal events, so reducing the room for improvement due to *n*−3 PUFA treatment, and, on the other hand, may also reduce the transferability of OMEGA results to the treatment of post-MI patients that, to date, still appear substantially different in clinical practice (Kotseva et al., 2009a,b).

The results of Alpha Omega Trial have been published a few weeks after the publication of OMEGA (Kromhout et al., 2010). Alpha Omega was a multicenter, double-blind, placebo-controlled trial testing the effects of *n*-3 PUFA on the rate of cardiovascular events among 4837 patients, 60 through 80 years of age, who have had a MI. Patients were randomly assigned to use for 40 months one of four types of margarine enriched with marine *n*−3 PUFA with a targeted additional daily intake of (a) 400 mg of a combination of EPA and docosahexaenoic acid (DHA); (b) plant-derived *n*−3 PUFA, with a targeted additional daily intake of 2 g of alpha-linolenic acid (ALA); (c) marine (400 mg/day) plus plant-derived (2 g/day) *n*−3 PUFA; (e) placebo. Neither marine- nor plant-derived *n*−3 PUFA reduced the primary endpoint, i.e., the rate of major CV events, including fatal and non-fatal CV events and cardiac interventions (hazard ratio, HR with EPA–DHA, 1.01; 95% CI: 0.87–1.17; $P = 0.93$; HR with ALA, 0.91; 95% CI: 0.78–1.05; $P = 0.20$). *n*−3 PUFA did not reduce the secondary endpoints, including ventricular arrhythmia-related events (SCD, fatal and non-fatal cardiac arrest, and placement of implantable cardioverter–defibrillators; HR with EPA–DHA, 0.90; 95% CI: 0.65–1.26; $P = 0.55$; HR with ALA, 0.79; 95% CI: 0.79–1.19; $P = 0.16$). Alpha Omega was a factorial trial and therefore only the two-way analyses comparing patients who received EPA–DHA to those not receiving marine *n*−3 PUFA as well as comparing patients who received ALA to those not receiving plant-derived *n*−3 PUFA were published. In the former case, 2404 patients with a mean daily supplementation of EPA-DHA of 400 mg plus a mean daily supplementation of ALA of 1008 mg was compared to 2433 patients with a mean daily supplementation of ALA of 984 mg.

In the latter case, 2409 patients with a mean daily supplementation of EPA-DHA of 201 mg plus a mean daily supplementation of ALA of 2000 mg was compared to 2428 patients with a mean daily supplementation of EPA-DHA of 196 mg. In other words, the effect of $n-3$ PUFA was not compared with a pure "placebo." The assessment of the effect of $n-3$ PUFA was likely to be biased because all the patients were receiving $n-3$ PUFA and in the case of marine $n-3$ PUFA the comparison was between two groups receiving approximately a total of 1.4 vs. 1.0 g of $n-3$ PUFA. The administration of $n-3$ PUFA in all patients of the Alpha Omega trial has made the results difficult to interpret and might have obscured the antiarrhythmic effect of $n-3$ PUFA if a threshold for the antiarrhythmic effect truly exists (de Lorgeril et al., 1994; Leaf, 1999; Zatonski et al., 2008). More recently the results of SU.FOL.OM3 study have been published (Galan et al., 2010). Among 1863 patients with CHD, neither a treatment with $n-3$ PUFA (600 mg/day), nor treatment with B vitamins was associated with a significant effect on the occurrence of hard coronary events, a composite endpoint including sudden death, after a mean follow-up of 4.2 ± 1.0 years. No information on sudden death was provided in this study.

HEART FAILURE

The GISSI-Prevenzione results showed that systolic dysfunction was associated with elevated risk of SCD and with a consistent benefit from $n-3$ PUFA (Macchia et al., 2005). This was the core hypothesis of the GISSI-HF Trial, a randomized, placebo-controlled trial investigating the efficacy of $n-3$ PUFA (850–882 mg EPA and DHA daily as ethyl ester compared to placebo) in 6975 patients who had clinical evidence of HF, classified as NYHA class II–IV, followed for a median of 3.9 years (Tavazzi et al., 2004, 2008). Statistically significant risk reductions in the two co-primary endpoints were observed in $n-3$ PUFA treated patients: 9% (adjusted HR 0.91; 95% CI: 0.833–0.998; $P = 0.041$) and 8% (adjusted HR 0.92; 95% CI: 0.849–0.999; $P = 0.009$) for mortality and mortality plus admission to hospital for CV reason, respectively (**Table 2**). It might be argued that the benefit showed by $n-3$ PUFA in HF patients is only marginal, being modest as the amount of relative risk reduction was modest. Actually, due to the high mortality and hospitalization rates in HF patients, the relative risk reduction observed in GISSI-HF can be translated into an absolute benefit of 18 deaths avoided and 17 prevented CV hospitalizations for every 1000 patients treated for 3.9 years with $n-3$ PUFA, i.e., a not trivial benefit as compared to statins and ACE-inhibitors in high-risk populations (Yusuf et al., 2000; Heart Protection Study Collaborative Group, 2002). The main study results were consistent with those of the secondary analyses, which were predefined in the study protocol, for (a) baseline characteristics, (b) secondary outcomes, and (c) per-protocol analysis in the 4994 patients who were compliant to experimental treatments. Worsening of HF and presumed arrhythmic death accounted for 62% of all deaths and were lower in the $n-3$ PUFA as compared to placebo group. Almost half of the absolute reduction of risk of death due to $n-3$ PUFA treatment was attributable to the reduction of ventricular arrhythmias (mortality: 0.9% out of 1.8%; first CV hospitalization: 1.0% out of 1.7%). Moreover, the rate of total major ventricular arrhythmias, defined as the occurrence of arrhythmic death or hospitalization due to ventricular arrhythmias, was significantly reduced early after the start of treatment in the 3 PUFA group (Marchioli et al., submitted). These findings support the idea that also in the clinical setting of HF $n-3$ PUFA may exert their antiarrhythmic effect. In addition, the benefit in both co-primary endpoints (mortality and hospitalization) suggests that $n-3$ PUFA might positively affect the pathophysiologic mechanisms leading to the progression of HF (Marchioli et al., 2009; Ghio et al., 2010). GISSI-HF Trial had some weaknesses and limitations. The patients enrolled in the GISSI-HF trial were already treated with guideline-based therapies for HF, so the benefit of $n-3$ PUFA can be considered as additive to the one determined by recommended treatments. Moreover, GISSI-HF trial adopted a pragmatic strategy, representing what happens in real-world settings of clinical practice. This assures the transferability of the GISSI-HF results to all patients with HF and suggest to not consider a limitation the apparent high percentages of patients who were not fully compliant with experimental treatments (by the end of the study 28.7% in the $n-3$ PUFA group and 29.6% in the placebo group). Although the observed benefit was lower than expected, it is comparable to the one obtained by the use of chronic preventive treatments in other clinical settings (Yusuf et al., 2000; Heart Protection Study Collaborative Group, 2002). On the other side, the high percentage of fully treated patients may have left limited room to further improvement. The low number of patients with preserved left ventricular ejection fraction (<10%) may be partially considered as a limitation since it precluded to perform a reliable assessment of the effect of $n-3$ PUFA in this subgroup of patients.

PATIENTS WITH IMPLANTABLE CARDIOVERTER DEFIBRILLATOR

Few studies evaluated the antiarrhythmic effect of $n-3$ PUFA in patients with ICD, reporting mixed results. Two hundred patients with a recent episode of sustained ventricular tachycardia (VT) or ventricular fibrillation (VF) and implanted ICD were randomly assigned to receive $n-3$ PUFA, 1.8 g/day, or placebo in trial by Raitt et al. (2005) At a median follow-up of 718 days, $n-3$ PUFA did not prevent episodes of VT or VF. Overall, $n-3$ PUFA treatment was associated with a trend toward a higher incidence of the primary endpoint (time to ICD therapy for VT/VF; $P = 0.19$), a significant increased rate of recurrent episodes of VT/VF ($P < 0.001$) and, in patients with VT as the qualifying entry rhythm, a significant increase in the primary endpoint ($P = 0.007$).

One year after this first trial, the results of The Study on Omega-3 Fatty acids and ventricular Arrhythmia (SOFA) have been published (Brouwer et al., 2006). The SOFA trial was a randomized, parallel, placebo-controlled, double-blind study evaluating the effect of $n-3$ PUFA 2 g/day for a median period of 356 days, on appropriate ICD intervention for VT or VF, or all-cause death in 546 patients with ICD and prior documented malignant VT or VF. No statistically significant protective effect in $n-3$ PUFA group was shown (HR 0.86; 95% CI: 0.64–1.16; $P = 0.33$).

As to the results of these two trials, some aspects of study design have to be considered. The first point is the type of population enrolled in these studies. Approximately 23% of patients in the trial of Raitt et al. (2005), and the 30% in SOFA had no history

Table 2 | Recent large clinical trials on the antiarrhythmic effects of n-3 PUFA in patients with HF.

Study	Patients	Number	Treatment daily dose	Control	Follow-up	Endpoints	Events (%) control goup	RR (95% CI)	Study power for		
									30% RRR	20% RRR	15% RRR
GISSI-HF (Tavazzi et al., 2008)	clinical evidence of HF (NYHA class II–IV)	7046	*n*−3 PUFA 1 g/day (850–882 mg EPA and DHA in the average ratio of 1:1·2; *n* = 3494)	Placebo (*n* = 3481)	Median 3.9 years	**All-cause death**	1014 (29.1)	0.91 (0.833–0.998)[a] †	>99	>99	98
						All-cause death or Hospital admission for CV reasons	2053 (58.9)	0.92 (0.849–0.999)[b] ‡	>99	>99	>99
						CV death	765 (22.0)	0.90 (0.81–0.99)†	>99	>99	93
						SCD	325 (9.3)	0.93 (0.79–1.08)†	99	80	53
						Hospital admission	2028 (58.3)	0.94 (0.88–1.00)†	>99	>99	>99
						For CV reasons	1687 (48.5)	0.93 (0.87–0.99)†	>99	>99	>99
						For HF	995 (28.6)	0.94 (0.86–1.02)	>99	>99	98
						All-cause death or Hospital admission (any reason)	2202 (63.3)	0.94 (0.89–0.99)*	>99	>99	>99
						Fatal or non-fatal MI	129 (3.7)	0.82 (0.63–1.06)	74	39	23
						Fatal or non-fatal stroke	103 (3.0)	1.16 (0.89–1.51)	64	32	19
						Fatal MI	25 (0.7)		17	8	6
						Death for HF	332 (9.5)		>99	80	55
						Presumed arrhythmic death	304 (8.7)		98	76	50

*EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; primary endpoints in bold; RR, relative risk; CI, confidence risk; RRR, relative risk reduction; [a]95.5% CI; [b]99% CI; *P < 0.10, †P < 0.05, ‡P < 0.01.*

of CHD. The best evidence for the antiarrhythmic action of *n*−3 PUFA has been produced by experimental work performed in ischemia-mediated animal models and in clinical trials carried out in post-MI patients, in which ischemia-triggered arrhythmias may be predominant. Consequently, it has been hypothesize that *n*–3 PUFA may have a more favorable effect in patients in whom the specific clinical setting and underlying arrhythmogenic mechanisms of initiation and propagation may be more susceptible to the antiarrhythmic effects of *n*–3 PUFA (Leaf et al., 2003). The second aspect is the primary endpoint used in ICD trials, i.e., VT or VF. It certainly may address the influence of *n*–3 PUFA on the risk of ventricular arrhythmias, however it might not be the ideal surrogate for the risk of sudden death. Although VT may frequently degenerate in VF and consequently in a sudden death, the spontaneous resolution of this ventricular arrhythmia is not uncommon. We cannot exclude that the treatment with *n*–3 PUFA might increase this favorable event, impossible to be showed in patients with ICD because of the device intervention. Moreover caring physicians were allowed to program ICD as more appropriate for their patients, so it has to be taken into consideration that this may represent a possible bias. Another aspect to be considered is the statistical power of the two studies. The power of trial by Raitt was significantly weakened by the low event rate observed in the placebo group as compared with the expected one. In addition, the observed reduction of events was lower than expected (**Table 3**). The total event rate of the primary endpoint observed in SOFA (33%) was close to the expected one (35%), but the observed efficacy of *n*–3 PUFA was lower than the expected one that was used for sample size calculation. Accordingly, SOFA had a 69% power to detect a 30% event reduction, so it is not surprising that this study did not detect a significant reduction in the primary endpoint (**Table 3**).

At variance with these two studies, in the FAAT study (Leaf et al., 2005) the administration of 2.6 g/day of *n*–3 PUFA vs. placebo in 402 patients at high-risk for fatal ventricular arrhythmias showed a trend toward a reduction of the primary endpoint (risk reduction of −28% of the time to the first ICD event for VT or VF, or death from any cause; $P = 0.057$). Although the choice to include all-cause death in the primary endpoint might be criticized, when probable and definite episodes of VT or VF were used as outcome measure, a statically significant relative risk reduction of 31% was obtained ($P = 0.033$; Leaf et al., 2005).

Two meta-analyses of data collected in these three trials did not support a protective effect of *n*−3 PUFA on cardiac arrhythmias. The first meta-analysis showed a significant heterogeneity between trials, and no effect of *n*−3 PUFA on the relative risk of ICD discharge (RR 0.93; 95% CI: 0.70–1.24; Jenkins et al., 2008). The second meta-analysis, confirmed the existence of a considerable heterogeneity between the trials, and showed no convincing protective effect of *n*−3 PUFA on time to first confirmed VF or VT combined with death (RR 0.90; 95% CI: 0.67–1.22), although the HR for the subgroup of patients with coronary artery disease at baseline tended toward a protective effect (RR 0.79; 95% CI: 0.60–1.06; Brouwer et al., 2009).

The results of a GISSI-HF sub-study assessing the antiarrhythmic effect of *n*−3 PUFA in 566 patients with HF enrolled who had received an ICD for secondary or primary prevention of VF or VT have been recently published (Finzi et al., 2011). The primary endpoint (defined as time to first appropriate ICD discharge for VT/VF; adjusted HR = 0.80, 95% CI: 0.59–1.09, $P = 0.152$), and the number of ICD discharges were not significantly altered ($P = 0.30$) by *n*−3 PUFA treatment.

The meta-analysis of these four trials on *n*−3 PUFA in patients with implanted ICD did not demonstrate a significant antiarrhythmic effect of *n*−3 PUFA (OR 0.82; 95% CI: 0.67–1.01; $P = 0.06$; **Figure 1**).

PATIENTS AT RISK OF SUPRAVENTRICULAR TACHYARRHYTHMIAS

Although results of observational studies suggested positive effects of *n*−3 PUFA in reducing the number of episodes as well as the burden of atrial tachyarrhythmia (Biscione et al., 2005), randomized control studies showed discrepant results (**Table 4**). An open-label, prospective, randomized, controlled trial with parallel groups, tested the effect of 2 g/day of *n*−3 PUFA on the development of AF in the postoperative period in 160 patients undergoing coronary artery by-pass grafting (CABG; Calo et al., 2005). Its results showed that *n*−3 PUFA administration before and during hospitalization substantially reduced the incidence of postoperative AF by 54.4% and was associated with a shorter hospital stay as compared the control group. In 199 patients with persistent AF treated with amiodarone and a renin-angiotensin-aldosterone system inhibitor, the addition of *n*−3 PUFAs 2 g/day improved the probability of the maintenance of sinus rhythm at 1 year after direct current cardioversion (Nodari et al., 2011). At variance with the previous study, a prospective, randomized, double-blind, placebo-controlled, parallel-group multicenter trial involving 663 outpatient participants with previous confirmed symptomatic paroxysmal ($n = 542$) or persistent ($n = 121$) AF reported no difference between treatment groups (*n*−3 PUFA 8 g/day or placebo for the first 7 days; *n*−3 PUFA 4 g/day or placebo thereafter through week 24) for recurrence of symptomatic AF in the paroxystic AF stratum (HR, 1.15; 95% CI: 0.90–1.46; $P = 0.26$), in the persistent AF stratum (HR, 1.64;95% CI: 0.92–2.92; $P = 0.09$), and in both strata combined (HR, 1.22; 95% CI: 0.98–1.52; $P = 0.08$; Kowey et al., 2010).

A recent systematic review and meta-analysis of published randomized trial regarding *n*−3 PUFA supplementation for AF prevention on 1955 patients showed a significant heterogeneity among the studies [$P = 0.002$, $I(2) = 65.0\%$] and did not find a significant reduction in the risk for AF (OR 0.81, 95% CI: 0.57–1.15; $P = 0.24$; Liu et al., 2011).

The discrepant results on the benefit of *n*−3 PUFA in preventing supraventricular tachyarrhythmia in the above discussed trials may be due to several reasons. The role of several patient characteristics were probably underestimated.

Left atrial dimension, AF duration, recurrent episodes of AF, which reduced the possibilities of restore or maintain a stable sinus rhythm (Suzuki et al., 2011; Pisters et al., 2012), were nor included among inclusion criteria neither in subgroup analysis in all but one of the studies. Nodari et al. (2011) showed that the main correlates of AF recurrence were, other than *n*−3 PUFA, the duration of AF before randomization, left atrial dimension, and left ventricular ejection fraction, suggesting that atrial remodeling may play

Table 3 | Recent large clinical trials on the antiarrhythmic effects of n-3 PUFA in patients with ICD.

Study	Patients	Number	Treatment daily dose	Control	Follow-up	Endpoints	Events (%) control goup	RR (95% CI)	Study power for		
									30% RRR	20% RRR	15% RRR
Leaf et al. (2005)	Subjects with an ICD because of a history of cardiac arrest, sustained VT, or syncope with inducible, sustained VT or VF during electrophysiologic studies.	402	*n*−3 PUFA 4.0 g/day (total dose of EPA plus DHA of 2.6 g; *n* = 200)	Olive oil (*n* = 202)	12 months	First ICD event for VT or VF confirmed by stored electrograms or death from any cause	78 (39)	0.67 (0.47–0.95)†	66	33	20
Raitt et al. (2005)	Patients with an ICD and a recent episode of sustained VT or VF	200	Fish oil 1.8 g/day (consisting of 42% EPA) and 30% DHA; *n* = 100)	Olive oil (73% oleic acid, 12% palmitic acid, 0% EPA/DHA; *n* = 100)	718 days	First episode of VT/VF leading to ICD therapy 6 months	6 months 36 (SE 5%)		32	15	10
						12 months	12 months 41 (5%)		39	18	11
						24 months	24 months 59 (5%)	1.26 (0.88–1.86)	66	33	20
SOFA (Brouwer et al., 2006)	Subjects who had either an ICD or were about to receive one and at least 1 confirmed VT or VF in the preceding year	546	Fish oil 2 g/day (four capsules containing 961 mg of omega-3 PUFAs (464 mg EPA, 335 mg DHA, and 162 mg other omega-3 PUFAs; *n* = 273)	Placebo containing 2 g of high-oleic acid sunflower oil. (*n* = 273)	356 days	**Sustained ICD intervention or death from any cause**	90 (33)	0.86 (0.64–1.16)	69	36	21
						Death from any cause	14 (5)		10	5	4
						Death for Cardiac cause	13 (5)		10	5	4
						ICD intervention for first event	81 (30)	0.89 (0.65–1.22)	64	32	19
						MI	3 (1)		<1	<1	<1
Finzi et al. (2011)	Patients with HF and an ICD for secondary or primary prevention of VF or VT	566	*n*−3 PUFA 1 g/day (850–882 mg EPA and DHA in the average ratio of 1:1·2; *n* = 278)	Placebo (*n* = 288)	928 days (median)	**Appropriate ICD intervention for major arrhythmias**	82 (28.5)	0.80 (0.59–1.09)	54	36	21

VT, ventricular tachycardia; VF, ventricular fibrillation; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; primary endpoints in bold; RR, relative risk; CI, confidence risk; RRR, relative risk reduction; †P < 0.05.

Table 4 | Recent large clinical trials on the antiarrhythmic effects of n-3 PUFA in patients with AF.

Study	Patients	Number	Treatment daily dose	Control	Follow-up	Endpoints	Events (%) control goup	RR (95% CI)	Study power for		
									30% RRR	20% RRR	15% RRR
Calo et al. (2005)	Patients undergoing CABG	160	*n*−3 PUFA 2 g/day (850–882 mg EPA and DHA in the average ratio of EPA/DHA 1:2 in each 1-g capsule; *n* = 79)	Usual care (*n* = 81)	Days 8.2 ± 2.6 c 7.3 ± 2.1 t	**Occurrence of AF**	27 (33.3)	0.32 (0.10–0.98)[†]	23	11	7
						Hospital length of stay	8.2 ± 2.6 days				
Kowey et al. (2010)	Subjects with a confirmed diagnosis of symptomatic paroxysmal AF	663 (527 paroxysmal AF; 118 persistent AF)	*n*−3 PUFA 4 g/day (approximately 465 mg of EPA and 375 mg of DHA in each 1-g capsule). (*n* = 332; 258 parox AF; 64 pers AF)	Corn oil approximately 8 g/day (*n* = 269; 331 parox AF; 54 pers AF)	6 months	**First recurrence of symptomatic AF or flutter**	129 (48)	1.15 (0.90–1.46)	91	57	35
						Independent analysis[a]	129 (47)	1.19 (0.93–1.35)	90	55	34
						First recurrence of symptomatic AF or flutter[b]	136 (49)	1.15 (0.91–1.45)	92	58	36
						First recurrence of symptomatic AF (exclusive of flutter)	126 (47)	1.17 (0.91–1.49)	90	57	35
						Independent analysis[a]	126 (46)	1.22 (0.95–1.56)	89	54	33
						First recurrence of symptomatic or asymptomatic AF or flutter	149 (55)	1.12 (0.89–1.40)	96	68	44
						Independent analysis[a]	152 (55)	1.13 (0.90–1.42)	96	68	44
						First recurrence of symptomatic or asymptomatic AF (exclusive of flutter)	146 (54)	1.14 (0.90–1.43)	96	67	43
						Independent analysis[a]	149 (54)	1.15 (0.92–1.45)	96	67	43

(Continued)

Table 4 | Continued

Study	Patients	Number	Treatment daily dose	Control	Follow-up	Endpoints	Events (%) control goup	RR (95% CI)	Study power for		
									30% RRR	20% RRR	15% RRR
Nodari et al. (2011)	Persistent AF lasting ≥1 month and confirmed by ECG Holter monitoring and history of at least one relapse after previous successful cardioversion	199	*n*−3 PUFA 2 g/day (850–882 mg of EPA and DHA in the average ratio of EPA/DHA of 1.2 in each 1.0-g capsule (allowed range, 0.9–1.5; *n* = 100)	Olive oil 2 g/day (*n* = 99)	1 year	Probability of maintenance of sinus rhythm	56/99 (56.6)	**0.62 (0.52–0.72) §**	62	30	185

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; primary endpoints in bold; RR, relative risk; CI, confidence risk; RRR, relative risk reduction; †P < 0.05, §P < 0.001; [a]Values are based on the independent statistician's analyses (participants with antiarrhythmic drug use were not censored) using Cox proportional hazard model: log (hazard ratio) equals treatment plus region plus angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker plus statin.

[b]Sensitivity analysis using the original stratum at randomization.

a role in this clinical setting. Similarly to the case of drugs inhibiting renin-angiotensin system, clinical success in restoring sinus rhythm is likely to be achievable only before the underlying atrial remodeling may have gone too far to be reversed (Savelieva et al., 2011). Baseline plasma levels of *n*−3 PUFA may have influenced the antiarrhythmic effect of oral supplementation (Skuladottir et al., 2011), and the use of new-onset, postoperative AF may not be the ideal model to assess the effect of *n*−3 PUFA on new-onset AF.

To date, as Camm and Savelieva (2011) affirmed in their editorial comment to the Nodari's article, the antiarrhythmic effect of *n*−3 PUFA in AF has not been demonstrated in randomized clinical trial. A large scale randomized controlled trial testing the effect of *n*−3 PUFA on supraventricular arrhythmias in patients undergoing to cardiac surgery is currently ongoing (Mozaffarian et al., 2011). Its results will increase our knowledge on the antiarrhythmic effects as well as on tolerability of *n*−3 PUFA in this clinical setting.

REVIEWS AND META-ANALYSES

The effect of *n*−3 PUFA on cardiovascular events and SCD has been analyzed in some reviews and meta-analyses.

A Cochrane review (Hooper et al., 2004) and a meta-analysis (Hooper et al., 2006) did not find a benefit of *n*−3 PUFA administration. The Cochrane review (Hooper et al., 2004), which included 89 studies (48 randomized controlled studies and 41 cohort studies), found that *n*−3 PUFA did not cause a significant reduction in the risk of total mortality (RR 0.87; 95% CI: 0.73–1.03) or combined CV events (RR 0.95; 95% CI: 0.82–1.12). Considerable debate was generated by the choice of inclusion of the DART II (Burr et al., 2003) in the analysis, because of various limitations of the study and the difficulties occurring during its conduct (von Schacky et al., 2006; Graham et al., 2007; von Schacky and Harris, 2007). After excluding DART II from the Cochrane analysis, the reduction of total death became statistically significant (RR 0.83; 95% CI: 0.75–0.91), although the risk of CV events did not change (Hooper et al., 2004). More recently a review, at variance with the previous ones, pooled the effect on SCD of eight trials, comprising 20,997 patients, and showed that the treatment with *n*−3 PUFA was able to significantly reduce the incidence of SCD (RR 0.43; 95% CI: 0.20–0.91) in post-MI patients. In patients with angina the risk of SCD was increased (RR 1.39; 95% CI: 1.01–1.92) and overall, *n*−3 PUFA had no effect on cardiac death and all-cause mortality (RR 0.71 and 0.77; 95% CI: 0.50–1.00 and 0.58–1.01, respectively; Zhao et al., 2009). In 2010 a meta-analysis of 29 randomized controlled trials, comprising 35.144 patients, showed that *n*−3 PUFA treatment was not associated with a statistically significant decreased mortality (RR 0.88; 95% CI: 0.64–1.03) although the probability of some benefits was high (0.93; Filion et al., 2010). A recent meta-analysis, including 20.485 patients with a history of CVD, showed that *n*−3 PUFA supplementation did not reduce the risk of overall CV events (RR 0.99; 95% CI: 0.89–1.09), all-cause mortality, SCD, MI, HF, or transient ischemic attack and stroke (Kwak et al., 2012). About the results of this last meta-analysis several aspects deserve to be considered, e.g., most studies were very small, not designed to evaluate CV endpoints and with a short follow-up, the methodological choice of using

study	n-3 PUFA		placebo		weight %	Odds Ratio M-H, fixed, (95%CI)
	events	total	events	total		
Leaf [11]	44	200	66	202	25.6	0.58 (0.37-0.91)
Raitt [12]	51	100	41	100	10.0	1.50 (0.86-2.62)
SOFA [13]	75	273	81	273	29.4	0.90 (0.62-1.30)
GISSI-HF [14]	76	278	98	288	35	0.73 (0.51-1.04)
total	**246**	**851**	**286**	**863**	**100**	**0.82 (0.67-1.01)**

Heterogeneity: X^2=7.39, df =3 (P=0.06); I^2 =59

Test for overall effect: Z= 1.91 (P=0.06)

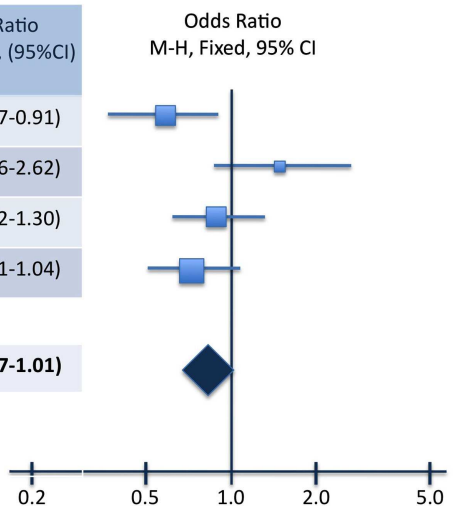

FIGURE 1 | Pooled analysis of the effect of n-3 PUFA on appropriate ICD intervention.

random-effects models in the primary analysis, and the exclusion of two large open-label trials (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico, 1999; Yokoyama et al., 2007).

CONCLUSION

The evidence available to date seem to suggest *n*−3 PUFA having an antiarrhythmic effects, but no firm conclusion can be drawn. The stronger evidence on positive effects of *n*−3 PUFA in reducing SCD came from studies including patients with CHD. In patients with symptomatic HF, *n*−3 PUFA seem to reduce significantly ventricular arrhythmias and SCD. Such effect on ventricular arrhythmia has not been convincingly confirmed so far by the results of trials in carriers of an ICD, though the meta-analysis of the studies in ICD patients is very suggestive. On the other side, the effect on recurrent/new-onset AF is still controversial. Many of the clinical trials conducted so had not significant results. The main cause of this "failure" is the inadequate power of the studies due to either low rate of events or the overoptimistic expectations of the benefit of *n*−3 PUFA used for sample size calculation. Serum baseline levels of *n*−3 PUFA and different doses may have contributed to such results.

In conclusion, the still debated antiarrhythmic effect of *n*−3 PUFA needs to be investigated in larger and/or more efficient trials than those conducted so far. On this regard it is possible that a more accurate selection of patients, e.g., evaluating relevant biological parameters and markers (type and length of AF, left atrial dimensions, plasma and cellular *n*−3 PUFA levels, systemic inflammatory status, or oxidative stress), a standardization in the device programming in ICD patient populations, and a "realistic" sample size calculation might answer some of the still open question on the antiarrhythmic effect of *n*−3 PUFA.

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Fish, marine *n*−3 fatty acids, and atrial fibrillation – experimental data and clinical effects

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Marine *n*−3 polyunsaturated fatty acids (PUFA) may have beneficial effects in relation to atrial fibrillation (AF) with promising data from experimental animal studies, however, results from studies in humans have been inconsistent. This review evaluates the mechanisms of action of marine *n*−3 PUFA in relation to AF based on experimental data and provides a status on the evidence obtained from observational studies and interventional trials. In conclusion, there is growing evidence for an effect of marine *n*−3 PUFA in prevention and treatment of AF. However, further studies are needed to establish which patients are more likely to benefit from *n*−3 PUFA, the timing of treatment, and dosages.

Keywords: atrial fibrillation, fish, marine *n*−3 polyunsaturated fatty acids

INTRODUCTION

Faced with the growing epidemic of atrial fibrillation (AF), there is an unmet need for preventive measures as well as better treatment of this disorder. Some of the advances in recent years have been on rhythm control to restore sinus rhythm, but the development of effective and safe drugs for restoring sinus rhythm has been difficult, trying to balance beneficial anti-arrhythmic effects with risk of pro-arrhythmic adverse effects. Invasive intervention by catheter ablation techniques and surgery may be more effective, but is an option mostly for highly symptomatic patients, given the risk of peri-procedure complications and high costs. Fish consumption and intake of marine *n*−3 polyunsaturated fatty acids (PUFA), with suggested anti-arrhythmic effects in relation to malignant ventricular arrhythmias, is safe and with few side-effects.

This review will discuss the mechanisms of action of marine *n*−3 PUFA in relation to AF based on experimental data and provide a status on the evidence of the effect of marine *n*−3 PUFA in AF as obtained from observational studies and interventional trials.

BACKGROUND

FISH, MARINE *n*−3 PUFA, AND CARDIAC DISEASE

In early studies of Greenland natives, Bang, and Dyerberg found a lower risk of death from coronary heart disease (Dyerberg et al., 1978). The Greenland Eskimos were living on a diet consisting largely on whales, seals, and fish, thus consuming around 10–14 g/day of marine *n*−3 PUFA, whereas most western populations consume less than 0.5 g/day (De Caterina, 2011). Several epidemiological studies have confirmed the finding in Greenland Eskimos, although data are not entirely consistent (Bjerregaard et al., 2010; De Caterina, 2011; Mozaffarian and Wu, 2011). Marine *n*−3 PUFA may affect the risk of cardiovascular disease by a long list of mechanisms including a lowering of triglycerides, a reduction in blood pressure, together with antithrombotic and anti-inflammatory effects (De Caterina, 2011; Mozaffarian and Wu, 2011). Furthermore, in some studies marine *n*−3 PUFA have lowered the risk of sudden cardiac death (De Caterina, 2011; Mozaffarian and Wu, 2011) and in turn, these observed effects on ventricular tachyarrhythmia have lead to research into potential effects of *n*−3 PUFA on atrial rhythm disturbances including AF.

MARINE *n*−3 PUFA

Polyunsaturated fatty acids are divided into *n*−3 (omega-3) and *n*−6 according to the position of the first double bond. Both groups are essential fatty acids, and therefore the content in the human body is (almost) fully dependent on dietary intake. The *n*−3 PUFA family consists of alpha-linolenic acid (ALA) derived from plants and the marine *n*−3 PUFA consisting of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA). Marine *n*−3 PUFA are most abundant in fatty fish and in the liver of lean fish (Calder, 2012). In addition to their dietary intake, EPA, DHA, and DPA can be synthesized endogenously from ALA but only to a very limited extent (Burdge and Calder, 2005). The biologically most important *n*−3 PUFA are thought to be EPA and DHA, while very little is known about the effect of DPA.

ATRIAL REMODELING

Atrial remodeling is defined as any persistent change in atrial structure or function (Nattel et al., 2008) that may occur either as a substrate induced by another underlying condition or secondary to AF itself. For AF to develop, some fundamental arrhythmia mechanisms are required; both a suitable substrate and some form of triggering activity, and these, in turn, facilitate and initiate reentry of electrical impulses, thereby maintaining AF (Nattel et al.,

2008). Atrial remodeling can increase the potential for ectopic and reentrant activity by a variety of mechanisms (Nattel et al., 2008).

Ectopic firing provides a trigger for reentry, but may also in itself maintain AF in the case of a rapid ectopic firing focus (Nattel et al., 2008). Abnormalities in cellular Ca^{2+} handling can cause ectopic firing, e.g., because of delayed after-depolarizations related to Ca^{2+} overload.

Ischemia, inflammation, and dilation make atria more vulnerable to AF (Nattel et al., 2008). For reentry to be maintained, the cells must have regained their excitability when the impulse has traversed the circuit, and therefore reentry is favored by shortening of the refractory period (e.g., by rapid atrial activation in AF), slow conduction of the impulse (e.g., by changes in cellular and tissue structure including fibrosis), and longer distance of the impulse circuit (e.g., atrial enlargement). The fibrillatory activity may occur either because of an irregular atrial response to a single reentry circuit or because of multiple simultaneous functional reentry circuits (Nattel et al., 2008).

Atrial remodeling can be divided into two major groups according to whether it is related to a rapid atrial rate such as in AF, or caused by an underlying condition. Rapid atrial tachycardia results in shortening of the atrial refractory period (electrical remodeling), occurs within the first few days of AF, and is reversed within 1 week after reversion to sinus rhythm (Allessie et al., 2002). Secondly, AF also results in impaired contractility (contractile remodeling) of the atria after reversion to sinus rhythm and depending on the duration of AF, and recovery is slower lasting days to months (Allessie et al., 2002). Atrial structural remodeling refers to structural changes in the atria and may occur either during prolonged atrial tachycardia or caused by different cardiac pathologies such as hypertensive heart disease, heart failure, myocardial infarction, and cardiomyopathy. It includes histological changes in the atrial myocytes in terms of dedifferentiation, and the associated atrial fibrosis causes intra-atrial conduction disturbances (Allessie et al., 2002). AF increases dramatically with older age, which is likely in part mediated by age-related fibrosis, changes in conduction, and increased likelihood of block (Dun and Boyden, 2009).

METHODS

SEARCH FOR LITERATURE

We searched PubMed using the MesH terms "AF" and ("Seafood," "Fatty Acids, Omega-3," "Fish Oils," "Fishes," or "Dietary Fats") and EMBASE using the terms "heart atrium fibrillation" and ("omega-3 fatty acid," "sea food," "fish oil," or "exp fish"). Reference lists from identified reports were checked for additional publications. Criteria for consideration were publications in English published until February 2012. All identified abstracts were assessed, and relevant published reports were identified. We discarded results only published as abstracts.

MARINE *n*−3 PUFA AND CARDIAC ARRHYTHMIAS

MECHANISMS OF ACTION

The effects of marine *n*−3 PUFA in relation to ventricular arrhythmia have been extensively studied whereas few have studied the effects on atrial myocytes. For this reason, some mechanisms of action related to ventricular arrhythmia are summarized in order to further suggest possible effects of *n*−3 PUFA on atrial myocytes.

As recently reviewed (Saravanan et al., 2010b; Mozaffarian and Wu, 2011), *n*−3 PUFA have beneficial effects on various cardiovascular risk factors, inflammation, and effects related to arrhythmia via several mechanisms including effects on cell and organelle membrane structure and function; ion channels and electrophysiology; and nuclear receptors and transcription factors.

Anti-arrhythmic effects of a diet rich in marine *n*−3 PUFA were studied in a rat model by McLennan (1993) where these fatty acids were shown to prevent ventricular fibrillation in rats, and, in similar experiments in marmoset monkeys, they increased the thresholds for ventricular fibrillation (McLennan et al., 1993).

Likewise, subsequent studies of intravenous infusion of *n*−3 PUFA in a dog model of ventricular arrhythmia showed that ventricular fibrillation was much more difficult to elicit after infusion of marine *n*−3 PUFA (Billman et al., 1999). In a series of *in vitro* studies, Leaf et al. (Leaf et al., 2005; Xiao et al., 2005) demonstrated anti-arrhythmic effects of marine *n*−3 PUFA. Thus, *n*−3 PUFA affect numerous ion channels such as inhibitory effects on the inward sodium current (I_{Na}) and L-type inward calcium current ($I_{Ca,L}$) which may be important for their anti-arrhythmic effects (Leaf et al., 2005). *n*−3 PUFA affect the I_{Na} by shifting the steady-state inactivation to hyperpolarized potentials and thereby prolonging the refractory period (Xiao et al., 1998, 2000). Inhibition of $I_{Ca,L}$ by *n*−3 PUFA (Xiao et al., 1997) prevents triggering activity from after-potential discharges caused by excessive cytosolic Ca^{2+} fluctuations (Leaf et al., 2005). Also, *n*−3 PUFA have electrical stabilizing effects on myocytes as shown *in vitro* (Kang and Leaf, 1996) where myocytes added EPA showed reduced spontaneous beating rate and required higher electrical stimulation to contract (Leaf et al., 2005).

ACUTE VS. CHRONIC EFFECTS

n−3 PUFA may principally operate in at least two different ways, either circulating in the blood or after incorporation into cells. Studies on circulating *n*−3 PUFA typically have used experimental designs where *n*−3 PUFA is added intravenously or *in vitro* in cellular studies and may thus be termed "acute effects," whereas "chronic effects" reflect dietary exposure over time where *n*−3 PUFA is incorporated into cellular membranes in addition to a steady-state of circulating *n*−3 PUFA (comparatively less than when administered intravenously). The period to full incorporation of *n*−3 PUFA has been reported to be 28 days for myocardium content of DHA in rats (Owen et al., 2004), 12 weeks for incorporation of EPA and DHA in right atrium and left ventricle in dogs (Billman et al., 2010), and 30 days for DHA and EPA in right atrial appendage tissue in humans (Metcalf et al., 2007). Importantly, "acute effects" of circulating *n*−3 PUFA have been shown to be different from "chronic effects" of *n*−3 PUFA in cellular membranes, which include different effects on various ion channels as reviewed by Den Ruijter and Coronel (2009). For example, circulating *n*−3 PUFA inhibit I_{Na} and $I_{Ca,L}$, whereas *n*−3 PUFA incorporated into cellular membranes do not affect I_{Na}, but both inhibit $I_{Ca,L}$ and the reopening of the calcium channel at plateau potentials. Thus, when interpreting clinical studies on *n*−3 PUFA, it is important to distinguish between studies on acute intravenous and long-term dietary exposure.

FISH, MARINE *n*−3 PUFA, AND ATRIAL FIBRILLATION

POTENTIAL MECHANISMS

Among these general effects on cardiac arrhythmia, some mechanisms may be of particular interest in relation to AF. Some mechanisms underlying AF include triggering activity by rapidly firing focal ectopic sources (often from the pulmonary veins) and reentry mechanisms including functional reentry circuits (probably more predominant in early stages such as paroxysmal AF) and multiple-circuit reentry (probably related to more pronounced structural remodeling in patients with persistent AF). *n*−3 PUFA reduce triggering activity and may affect reentry mechanisms in ventricular myocytes (Den Ruijter et al., 2007), and similar effects may occur in atrial myocytes (Nattel and Van Wagoner, 2011). This is, however, yet to be established.

Also, marine *n*−3 PUFA affect risk factors for ischemic heart disease such as lowering of blood pressure and plasma triglycerides and have anti-inflammatory effects, as they compete directly with *n*−6 PUFA as substrates for inflammatory eicosanoids, with the *n*−3-derived leukotrienes and thromboxanes being less inflammatory than products derived from *n*−6 PUFA (Calder, 2006).

Thus, the effects of marine *n*−3 PUFA on AF may be via mechanisms related to atherosclerosis and ischemic heart disease, including anti-inflammatory effects as well as direct anti-arrhythmic effects on myocytes through effects on ion channels, electrical stabilizing effects, and fluidity of the cell membrane.

EXPERIMENTAL ANIMAL STUDIES

In an *in vitro* model, induction of asynchronous contractile activity by a β-adrenoceptor stimulus in rat atrial myocytes was reduced by addition of DHA and EPA, while cell membrane fluidity was increased (Jahangiri et al., 2000). Also, EPA reduced arrhythmogenesis in isolated rabbit pulmonary vein tissue via nitric oxide production (Suenari et al., 2011).

Electrical remodeling by reduction of atrial refractory period is an important early remodeling event that favors the development and maintenance of AF. In a model of stretch-induced vulnerability of AF, rabbits fed *n*−3 PUFA were less susceptible to induce and sustain AF and also had less stretch-induced shortening of atrial refractory period (Ninio et al., 2005). Infusion of *n*−3 PUFA significantly reduced the shortening of atrial refractory period in an experimental model on dogs receiving rapid atrial pacing (da Cunha et al., 2007), whereas this was not found in a different study on dogs fed *n*−3 PUFA for a longer duration (Sakabe et al., 2007). Effects on structural remodeling were investigated in a ventricular tachypacing model of congestive heart failure. Dogs fed *n*−3 PUFA developed less atrial structural remodeling in terms of atrial fibrosis and conduction abnormalities as well as shorter duration of burst pacing-induced AF (Sakabe et al., 2007). Similar results were found in rabbits fed a diet of purified EPA which showed suppression of atrial structural remodeling with less cardiac fibrosis, shorter duration of induced AF, as well as a less inflammatory profile in atrial and epicardial adipose tissues (Kitamura et al., 2011). Also, in a model with 2 weeks of simultaneous atrioventricular pacing, dogs fed *n*−3 PUFA had less AF inducibility and shorter episodes of AF, reduced conduction anisotropy in the left atrium, and prevention of pacing-induced increase in collagen turnover and collagen deposition in atrial appendage (Laurent et al., 2008). Furthermore, there was a beneficial effect on genes related to fibrosis, hypertrophy, and inflammation (Ramadeen et al., 2010).

In a model of post-operative AF in dogs fed *n*−3 PUFA for 3 weeks before excision of the left atrial appendage, AF was not inducible (0/7) compared to induction in four of six control animals. In addition, *n*−3 PUFA-treated animals had longer post-operative atrial effective refractory period, increased heart rate variability, and reduced atrial inflammation (Mayyas et al., 2011). Likewise, in a model of AF induced by sterile pericarditis, dogs fed *n*−3 PUFA for 4 weeks had less inflammation and reduced inducibility and maintenance of AF (Zhang et al., 2011). Finally, in a model of vagally induced AF, dogs fed *n*−3 PUFA were less vulnerable to develop AF (Sarrazin et al., 2007).

Given the multiple disposing factors for developing AF with older age in humans, the type of experimentally induced AF in different animal models is a major limitation for interpreting the results in addition to the inherent differences between species. Also, acute effects of *n*−3 PUFA are tested by *in vitro* or intravenous administration of *n*−3 PUFA, whereas chronic effects are investigated in the models where animals have been fed *n*−3 PUFA. However, while the reported effects of treatment in these models suggest that they may serve as a useful tool for investigating possible effects of *n*−3 PUFA in relation to AF, human studies are needed to establish whether marine *n*−3 PUFA are clinically useful in this population.

Taken together, data from animal studies on AF show convincing results with a substantial effect of *n*−3 PUFA, and for some parameters there is almost a complete reduction of the differences induced in the experimental models. Thus, the effects of *n*−3 PUFA in these studies include reduction in the shortening of atrial refractory period (electrical remodeling), reduction of atrial fibrosis (structural remodeling), reduction of post-operative AF (inflammation), and a reduction in vagally induced AF.

PRIMARY PREVENTION OF AF

A number of observational studies have addressed whether intake of fish and marine *n*−3 PUFA was associated with a lower risk of developing AF (**Table 1**). Promising data on fish consumption and prevention of AF were originally found in a prospective cohort from the Cardiovascular Health Study (Mozaffarian et al., 2004). The 4815 study participants were 65 years or older, and during 12 years of follow-up, 980 incident cases of AF occurred. Consumption of tuna or other broiled or baked fish was associated with a 28% lower risk of AF with intake one to four times per week (HR 0.72, 95% CI 0.58–0.91, $p = 0.005$), and 31% lower risk with intake ≥5 times per week (HR 0.69, 95% CI 0.52–0.91, $p = 0.008$), compared to intake less than once per month. In contrast, consumption of fried fish or fish sandwiches was not correlated to plasma *n*−3 PUFA levels and was suggestive of a higher risk of AF although not statistically significant. These types of fish primarily include lean fish and in addition, the method of cooking influences the fatty acid content as frying has been reported to affect the fat content whereas the formation of oxidized cholesterol products is further increased by roasting (Echarte et al., 2001). Similar results were found in a Finnish prospective cohort study from the Kuopio Ischemic Heart Disease Risk Factor Study (Virtanen et al., 2009)

Table 1 | Studies of primary prevention of AF by consumption of fish and marine *n*–3 PUFA.

Reference	Study population (mean age at entry)	*n*	Incident cases of AF	Study design	Exposure	Outcome	Follow-up	Main results
Mozaffarian et al. (2004), US	Cardiovascular Health Study, adults (73 years)	4815	980	Cohort study	Fish consumption based on diet information	Incident AF	12 years	28% lower risk of AF with intake 1–4 times per week of tuna or other broiled or baked fish (HR 0.72, 95% CI 0.58–0.91, $p = 0.005$), and 31% lower risk with intake ≥5 times per week (HR 0.69, 95% CI 0.52–0.91, $p = 0.008$) compared with <1 time per month
Frost and Vestergaard (2005), Denmark	Diet Cancer and Health Study, adults (56 years)	47949	556	Cohort study	*n*–3 PUFA consumption based on diet information	Incident AF	5.7 years	34% higher risk of AF was seen when comparing highest vs. lowest quintile of *n*–3 PUFA intake (HR 1.34, 95% CI 1.02–1.76), whereas no association was seen in quintiles 2, 3, and 4 (HR 0.86, 1.08, 1.01 respectively)
Brouwer et al. (2006), Holland	Rotterdam Study, adults (67 years)	5184	312	Cohort study	Fish and *n*–3 PUFA consumption based on diet information	Incident AF	6.4 years	A non-significant higher risk of AF comparing highest vs. lowest tertile of *n*–3 PUFA intake (HR 1.18, 95% CI 0.88–1.57)
Macchia et al. (2008), Italy	Patients after myocardial infarction (65 years)	3242 (215 in *n*–3 group)	471	Population study on registry data	Prescription database records on *n*–3 PUFA	Incident AF	1 year	Relative risk reduction of 81% in hospitalization for AF (HR 0.19, 95% CI 0.07–0.51)
Virtanen et al. (2009), Finland	Kuopio IHD Risk Factor Study, males (53 years)	2174	240	Cohort study	Serum *n*–3 PUFA as a measure of dietary consumption	Incident AF	17.7 years	35% lower risk of AF comparing highest vs. lowest quartile of *n*–3 PUFA (HR 0.65, 95% CI 0.44–0.96). DHA but not EPA was associated with a lower risk of AF (HR 0.62, 95% CI 0.42–0.92)
Berry et al. (2010), US	Women's Health Initiative, postmenopausal women (63 years)	44720	378	Cohort study	Fish consumption based on diet information	Incident AF at 3 and 6 year visit	6 years	A non-significant higher risk of AF for highest vs. lowest quartile of fish intake (OR 1.17, 95% CI 0.88–1.57)
Shen et al. (2011), US	Framingham Heart Study, adults (62 years)	4526	296	Cohort study	Fish consumption based on diet information	Incident AF	4 years	No association. In exploratory subgroup analyses, >4 servings of fish/week (5 cases and 21 individuals at risk) was significantly associated with higher AF risk compared with consumption of <1 serving of fish/week (HR 6.53, 95% CI 2.65–16.06, $p < 0.001$)

AF, atrial fibrillation; n–3 PUFA, marine n–3 polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexanoic acid; HR, hazard ratio; OR, odds ratio; CI, confidence interval.

where a total of 240 incident cases of AF occurred during 18 years of follow-up among 2174 men aged between 42 and 60 years. The highest quartile of total *n*−3 PUFA content in serum at baseline was associated with a 35% lower risk of AF compared to the lowest quartile (HR 0.65, 95% CI 0.44–0.96, p for trend = 0.07). Serum DHA (but not EPA) was associated with the risk of AF, with a 38% lower risk of AF comparing the highest and lowest quartiles (HR 0.62, 95% CI 0.42–0.92, p for trend = 0.02). This inverse association has, however, not been found in other cohort studies (Frost and Vestergaard, 2005; Brouwer et al., 2006; Berry et al., 2010; Shen et al., 2011). Thus, in the study by Frost and Vestergaard (2005), there was a significantly higher risk of AF when comparing the highest vs. the lowest quintile of *n*−3 PUFA consumption. In an exploratory analysis in the study by Shen et al. (2011), the highest intake of dark (fatty) fish was also associated with a higher risk of AF. Overall, these studies have shown no clear association between higher fish or *n*−3 PUFA intake and incident AF. Finally, an Italian registry-based study on prescription of fish oil supplements for patients discharged after a myocardial infarction showed that significantly fewer had AF within the next year in the group prescribed fish oil supplements (Macchia et al., 2008).

Differences exist between the studies that may explain some of these contradictory results. The participants in the study by Mozaffarian et al. (2004) were older compared to the other studies, and the effects of fish intake may be more effective in this age category. First, the background diet in the population as well as the available fish products differ between studies Next, the causes of AF may differ between the study populations, and therefore the susceptibility to the effects of marine *n*−3 PUFA may differ. Also, patients may be motivated to a higher fish intake, for instance because of symptomatic coronary heart disease, and this would select persons who have higher morbidity and a higher *a priori* risk of AF, which in turn would bias the results in the direction of a detrimental effect of intake. It may also be that the effect, if any, of fish and fish oils is not simply a linear dose–response but may instead have a different association such as threshold effect or even a U- or J-shaped effect with detrimental impacts at both very low and very high levels. If a threshold effect is indeed present, establishing this would be very much dependent on the distribution of intake in the study population. Larger studies would be helpful in clarifying these issues, e.g., by using continuous exposure measures such as cubic splines. Although primary prevention of AF is of major importance, it is not likely that it would be feasible to conduct a RCT since this would require a very large study. For this reason, exploring a possible effect on primary prevention will be much dependent on further epidemiological studies.

PREVENTION OF RECURRENCE OF AF AFTER CARDIOVERSION

Although primary prevention of AF is very important for reducing the AF burden in a population, it is more feasible to motivate patients for secondary prevention of AF, where there is a need for safe and well-tolerated treatment options. As this setting is different, with varying degrees of electrical and structural remodeling ongoing or finalized, an effect of treatment may work by different mechanisms compared to the setting of primary prevention where effects on cardiovascular risk factors such as hypertension may be more predominant. For this reason, it is important to consider the type of patients when comparing results from different studies.

Recently, a number of RCT has been reported (**Table 2**), of which two showed a beneficial effect of treatment with marine *n*−3 PUFA (Nodari et al., 2011; Kumar et al., 2012), whereas this was not found in three other studies (Kowey et al., 2010; Bianconi et al., 2011; Ozaydin et al., 2011).

Thus, in a study by Nodari et al. (2011), 199 patients with persistent AF who were treated with amiodarone and a renin–angiotensin–aldosterone system inhibitor were randomized to 1.7 g *n*−3 PUFA/day or placebo 4 weeks before electrical cardioversion. The primary endpoint was maintenance of sinus rhythm at 1-year follow-up, and of the *n*−3 PUFA-treated patients, 62% were still in sinus rhythm compared to 36% in the placebo group ($p < 0.0001$).

Also in favor of a beneficial effect of *n*−3 PUFA is a recent study by Kumar et al. (2012) involving 178 patients with persistent AF who were randomized to 1.8 g *n*−3 PUFA/day or placebo for at least 1 month prior to electrical cardioversion. Primary endpoint was recurrence of persistent AF defined as AF documented for at least 1 week. At 90 days, 39% in the *n*−3 PUFA group had AF recurrence compared to 78% of controls (HR 0.38, 95% CI 0.27–0.56, $p < 0.001$). At 1 year, 67% of *n*−3 PUFA-treated patients and 90% of controls had persistent AF ($p < 0.001$). Extending the findings by Nodari et al., *n*−3 PUFA were associated with a significant reduction in AF recurrence with or without concurrent anti-arrhythmic drugs. An additional finding was that increasing levels of DHA as a percentage of fatty acids in the phospholipid fraction of serum predicted a lower risk of AF recurrence (HR 0.59, 95% CI 0.42–0.83, $p = 0.003$), whereas this was not statistically significant for EPA (HR 0.9, 95% CI 0.8–1.1, $p = 0.08$).

Contrary to these findings, no effect of treatment was found in a large study by Kowey et al. (2010) in patients with paroxysmal AF ($n = 542$) or persistent AF ($n = 121$) without structural heart disease. The patients were in sinus rhythm at baseline and randomized to 6.7 g *n*−3 PUFA/day for 7 days and 4 g/day thereafter. There was no difference between treatment groups for recurrence of symptomatic AF in the paroxysmal stratum (HR 1.15, 95% CI 0.90–1.46, $p = 0.26$), in the persistent stratum (HR 1.64, 95% CI 0.92–2.92, $p = 0.09$), or in both strata combined (HR 1.22, 95% CI 0.98–1.52, $p = 0.08$). Importantly, almost half of AF recurrences occurred within 2 weeks of *n*−3 PUFA supplementation. In comparison with the studies by Nodari et al. (2011) and Kumar et al. (2012), it can be argued that the effect of *n*−3 PUFA may not be fully penetrant this early, since it takes about 30 days for full incorporation of *n*−3 PUFA into atrial myocytes (Owen et al., 2004; Metcalf et al., 2007). Other differences were that patients were not on anti-arrhythmic drug treatment, had no structural heart disease, and predominantly had paroxysmal AF, and therefore a lesser degree of atrial remodeling.

Likewise, no effect was found by Bianconi et al. (2011) in a study of 204 patients with persistent AF randomized to 2.6 g *n*−3 PUFA/day or placebo starting ≥7 days (mean 3 weeks) prior to cardioversion and 2 g/day thereafter. The primary endpoint was AF recurrence which occurred in 59% of the *n*−3 PUFA-treated patients and in 51% of controls ($p = 0.28$). Compared to the studies by Nodari et al. (2011) and Kumar et al. (2012), a lack of effect

Table 2 | Trials on marine *n*−3 PUFA and atrial fibrillation after cardioversion.

	Study population	*n*	Study design	Exposure	Start of exposure	Outcome	Follow-up	Monitoring AF events	Main results
Kowey et al. (2010), US	Patients with paroxysmal ($n=542$) or persistent ($n=121$) AF and no structural heart disease	663	RCT	6.7 g *n*−3 PUFA/day for 7 days, hereafter 3.4 g/day	After cardioversion	Recurrence of AF	6 months	Transtelephonic ECG monitoring twice weekly	No difference between treatment groups for recurrence of symptomatic AF in the paroxysmal stratum (HR 1.15, 95% CI 0.90–1.46, $p=0.26$) or in the persistent stratum (HR 1.64, 95% CI 0.92–2.92, $p=0.09$)
Bianconi et al. (2011), Italy	Patients with persistent AF	204	RCT	2.6 g *n*−3 PUFA/day before cardioversion, hereafter 1.7 g/day	≥7 days before cardioversion (mean 21 days)	Recurrence of AF	6 months	Transtelephonic ECG monitoring twice weekly the first 3 months and five clinical visits	No difference. AF relapsed in 56 (58.9%) of the *n*−3 group and in 47 (51.1%) of controls ($p=0.28$). The mean time to AF recurrence was 83 days in the *n*−3 group and 106 days in the placebo group ($p=0.29$)
Nodari et al. (2011), Italy	Patients with persistent AF and treated with amiodarone and a renin–angiotensin–aldosterone system inhibitor	199	RCT	1.7 g *n*−3 PUFA/day	≥28 days before cardioversion (mean 33 days)	Recurrence of AF	1 year	24-h Holter monitoring at month 1, 3, 6 and, 12 and a total of seven clinical visits	At 1-year follow-up, the probability of maintenance of sinus rhythm was significantly higher in the *n*−3 group (0.62, 95% CI 0.52–0.72) compared to controls (0.36, 95% CI 0.26–0.46, log-rank test $p<0.001$)
Ozaydin et al. (2011), Turkey	Patients with persistent AF	47	RCT	Amiodarone vs. amiodarone plus 2 g *n*−3 PUFA/day	After cardioversion	Recurrence of AF	1 year	24-h Holter monitoring at month 1 and 3. Clinical visits weekly first month and monthly hereafter. AF defined as ≥10 min	No difference
Kumar et al. (2012), Australia	Patients with persistent AF	178	Open-label randomized study	1.8 g *n*−3 PUFA/day	≥30 days before cardioversion (mean 56 days)	Recurrence of persistent AF	1 year	ECG at week 2 and 6 and hereafter every 3 months. Persistent AF defined as AF on 2 ECGs at least 1 week apart	At 90 days, 38.5% of omega-3 patients had AF recurrence compared to 77.5% of controls (HR 0.38, 95% CI 0.27–0.56, $p<0.001$). At 1 year, 67% of omega-3 patients and 90% of controls were in persistent AF ($p<0.001$). The reduction in AF was present both for patients with or without concurrent anti-arrhythmic drugs

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Table 2 | (Continued).

	Study population	*n*	Study design	Exposure	Start of exposure	Outcome	Follow-up	Monitoring AF events	Main results
Kumar et al. (2012), Australia	Patients with fish intake ≤1 per week and undergoing cardioversion of persistent AF or radiofrequency ablation of right atrial flutter	49	Single-blinded RCT	1.8 g *n*−3 PUFA/day	≥30 days before cardioversion (mean 70 days)	Parameters of left atrial appendage function immediately before and after conversion	None	n/a	In AF patients, the *n*−3 group had less mean decrease in emptying velocity (8 vs. 32%, $p = 0.02$), less mean decrease in appendage emptying fraction (−9 vs. 39%, $p = 0.01$), lower incidence of new or increased spontaneous echocardiographic contrast (11 vs. 62.5%, $p = 0.003$), and lower incidence of atrial mechanical stunning (33 vs. 69%, $p = 0.03$). Likewise, reductions were also found in patients with atrial flutter

AF, atrial fibrillation; n−3 PUFA, marine n−3 polyunsaturated fatty acids; HR, hazard ratio; OR, odds ratio; CI, confidence interval; RCT, randomized controlled trial; ECG, electrocardiogram.

of *n*−3 PUFA supplementation may be explained by the fact that the majority of patients had been treated for less than 30 days prior to cardioversion. Differences in the background diet may also be a contributory factor, since the DHA concentration in blood only increased by 25% which is lower than 1.8-fold in the study by Kumar et al. (2012), suggesting a smaller difference between the intervention group and the placebo group. Finally, a small study by Ozaydin et al. (2011) with *n*−3 PUFA started after cardioversion also found no difference between groups.

In support of an effect of *n*−3 PUFA supplementation in patients with persistent AF are the results of a study on 49 patients concerning atrial mechanical function after reversal to sinus rhythm (Kumar et al., 2011c). After reversion of persistent AF (external or internal cardioversion) or persistent right atrial flutter (radiofrequency ablation) to sinus rhythm, parameters of left atrial appendage function were compared immediately before and after reversion. Consumption of 1.8 g *n*−3 PUFA/day for ≥1 month prior to reversion was shown to attenuate atrial mechanical stunning after reversion of AF and right atrial flutter to sinus rhythm.

At this point, more studies are needed to establish whether the potential beneficial effect on persistent AF observed in some studies is valid. It seems important to consider ≥30 days pre-treatment with *n*−3 PUFA to allow for incorporation in cell membranes. Notably, an effect on top of treatment with both amiodarone and a renin–angiotensin–aldosterone system inhibitor has been reported.

PREVENTION OF POST-OPERATIVE AF

Patients undergoing heart surgery such as coronary artery bypass grafting (CABG) often develop AF after the operation. In this setting, AF is induced by the intervention, and the mechanism is probably mediated by the highly inflammatory state following surgery.

A number of RCT have examined the effects of *n*−3 PUFA in patients undergoing heart surgery, especially CABG (**Table 3**). Promising results were initially published by Calo et al. (2005) in a study of 160 patients undergoing CABG. Patients were randomized to 1.7 g *n*−3 PUFA/day or placebo ≥5 days prior to surgery and were followed until discharge. The primary endpoint was the development of post-operative AF, which was seen in only 15% in the *n*−3 PUFA group compared to 33% of controls ($p = 0.013$). In addition, the *n*−3 PUFA group were hospitalized for significantly fewer days than the controls (7.3 vs. 8.2 days, $p = 0.017$).

Some support for a beneficial effect of *n*−3 PUFA was also reported by Sorice et al. (2011) in a study of 201 patients randomized to on-pump or off-pump CABG and supplement with 1.7 g *n*−3 PUFA/day or placebo. A significant reduction in post-operative AF was found in on-pump CABG patients treated with *n*−3 PUFA (11.7 vs. 31.6%, OR 0.28, $p = 0.01$), whereas no difference was seen for *n*−3 PUFA in off-pump CABG patients (11.1 vs. 12.5%, OR 0.87, $p = 0.84$). Also, Farquharson et al. (2011) studied 194 patients undergoing CABG or valve operating procedures who were randomized to 4.5 g *n*−3 PUFA/day or placebo starting 3 weeks prior to surgery. There was a 37% reduction of post-operative AF in the intervention group, however not significant (OR 0.63, 95% CI 0.35–1.11), and a non-significant delay in

Table 3 | Trials on marine *n*−3 PUFA and post-operative atrial fibrillation.

	Study population	*n*	Design	Exposure	Start of exposure	Outcome	Follow-up	Monitoring AF events	Main results
Calo et al. (2005), Italy	Patients undergoing CABG	160	RCT	1.7 g *n*−3 PUFA/day	≥5 days before surgery	Post-operative AF	AF before discharge	Continuous ECG 4–5 days, hereafter daily ECG until discharge, AF defined as episodes lasting ≥5 min	Reduction in post-operative AF in the *n*−3 group [15.2% ($n = 12$)] compared to controls [33.3% ($n = 27$), ($p = 0.013$)]
Heidt et al. (2009), Germany	Patients undergoing CABG	102	RCT	Intravenous fish oil infusion 100 mg/kg/day	From admittance to hospital	Post-operative AF	AF before transfer from ICU	Continuous ECG while in ICU. AF defined as episodes >15 min	Post-operative AF occurred in 15 patients (30.6%) in the control and in 9 (17.3%) in the *n*−3 group ($p < 0.05$). After CABG, the *n*−3 patients had to be treated in the ICU for a shorter time than the control patients
Heidarsdottir et al. (2010), Iceland	Patients undergoing CABG and/or valvular repair	168	RCT	2.2 g *n*−3 PUFA/day	5–7 days before surgery (range 2–28 days)	Post-operative AF	AF before discharge (maximum 2 weeks)	Continuous ECG, AF defined as episodes ≥5 min	No difference
Saravanan et al. (2010a), UK	Patients undergoing CABG	103	RCT	1.7 g *n*−3 PUFA/day	≥5 days before surgery (mean 17 days)	Post-operative AF	AF before discharge	Continuous ECG for 5 days, hereafter daily ECG until discharge. AF defined as episodes ≥30 s	No difference
Mariscalco (2010), Italy	Patients undergoing CABG and/or valve surgery or other heart surgery	530 (only 84 in the *n*−3 PUFA group)	Prospective observational study	Comparing patients taking 1 g *n*−3 PUFA/day at admission with patients not taking supplements. No information on why patients were started on *n*−3 PUFA	Median 5 days (range 1–26 days)	Post-operative AF before discharge or during cardiac rehabilitation	AF during admission or rehabilitation	Continuous ECG while in hospital, weekly ECG during rehabilitation. AF defined as episodes >15 min	After propensity score analysis, pre-operative *n*−3 PUFA was independently associated with a 46% reduction in risk of AF before discharge (OR 0.54, 95% CI 0.31–0.92) while no difference was found for AF during rehabilitation

(Continued)

Table 3 | (Continued).

	Study population	*n*	Design	Exposure	Start of exposure	Outcome	Follow-up	Monitoring AF events	Main results
Farquharson et al. (2011), Australia	Patients undergoing CABG or valve procedures	194	RCT	4.5 g *n*−3 PUFA/day	3 weeks before surgery (median 21 days)	Post-operative AF	AF before discharge or maximum 6 days	Continuous ECG for minimum 3 days, hereafter daily ECG. AF defined as episodes lasting ≥10 min	There was a non-significant reduction in the *n*−3 group with 37% post-operative AF vs. 48% in the control group (OR 0.63, 95% CI 0.35–1.11), as well as a non-significant delay in time to onset of AF in the *n*−3 group (HR 0.66, 95% CI 0.43–1.01). A significant reduction in length of stay in the ICU was seen in the *n*−3 group (ratio of means 0.71, 95% CI 0.56–0.90)
Sorice et al. (2011), Italy	Patients randomized to on-pump or off-pump CABG	201	RCT	1.7 g *n*−3 PUFA/day or placebo, and randomized to on-pump or off-pump CABG (four groups)	≥5 days before surgery	Post-operative AF	AF before discharge	Continuous ECG for 4 days and daily ECG hereafter. AF defined as lasting ≥5 min	A significant reduction in post-operative AF was seen for on-pump CABG patients treated with *n*−3 PUFA (11.7 vs. 31.6%, OR 0.28, $p = 0.01$) whereas no difference for *n*−3 PUFA in off-pump CABG patients (11.1 vs. 12.5%, OR 0.87, $p = 0.84$)

AF, atrial fibrillation; n−3 PUFA, marine n−3 polyunsaturated fatty acids; HR, hazard ratio; OR, odds ratio; CI, confidence interval; RCT, randomized controlled trial; ECG, electrocardiogram; CABG, coronary artery bypass graft surgery; ICU, intensive care unit.

Table 4 | Other studies on marine *n*−3 PUFA and atrial fibrillation.

	Study population	*n*	Study design	Exposure	Duration of exposure	Outcome	Follow-up	Monitoring AF events	Main results
Biscione et al. (2005), Italy	Patients with dual-chamber pacemakers	40	Open-label serial intervention with *n*−3 PUFA	1 g *n*−3 PUFA/day	4 months	Number of episodes and burden of atrial tachyarrhythmia	n/a	Open-label serial intervention with *n*−3 PUFA and observation by pacemaker interrogation before treatment, after 4 months of treatment, and after 4 months of withdrawal	Reduction in number of episodes and burden of atrial tachyarrhythmia for the *n*−3 treatment period, compared to before and after. Pre-treatment period number of episodes 444 ± 1161 and the burden 3.89% of time; in the *n*−3 treatment period respectively 181 ± 436 (−59%, $p = 0.037$), and 1.06% (−67%, $p = 0.029$). After *n*−3 withdrawal, the episodes of atrial tachyarrhythmia increased to 552 ± 1717 ($p = 0.065$) and the burden to 2.69% ($p = 0.003$)
Patel et al. (2009), US	Comparison of patients taking fish oil supplements 1 month prior to ablation and during follow-up period vs. controls	258	Nested case–control study	≥0.665 g of fish oil	n/a	Recurrence of AF	Up to 60 months	Rhythm transmission three times daily first 5 months. 24-h Holter monitoring at 3, 6, 9, 12 months, and every 6 months hereafter.	Patients taking fish oil supplements had less early recurrence of AF within 8 weeks (27 vs. 44%, $p < 0.001$) as well as less late recurrence of AF after 8 weeks (23 vs. 32%, $p < 0.003$)
Kumar et al. (2011b), Australia	Patients undergoing pulmonary vein isolation	36	Unblinded, randomized study	1.8 g *n*−3 PUFA/day	≥30 days prior to procedure	Parameters of left atrial electrophysiology	None	n/a	The *n*−3 group exhibited prolonged pulmonary venous and left atrial effective refractory periods and decreased susceptibility to initiation AF from within the pulmonary veins
Kumar et al. (2011a), Australia	Patients undergoing electrophysiological study for supraventricular arrhythmias	61	Single-blinded RCT	1.8 g *n*−3 PUFA/day	≥30 days before procedure	Parameters of atrial electrophysiology	None	n/a	Chronic fish oil supplementation prolonged atrial refractoriness and reduced vulnerability to inducible AF

(Continued)

Table 4 | (Continued).

	Study population	*n*	Study design	Exposure	Duration of exposure	Outcome	Follow-up	Monitoring AF events	Main results
Skuladottir et al. (2011), Iceland	Patients from the RCT by Heidarsdottir undergoing CABG and who were randomized to *n*−3 PUFA or placebo 5–7 days prior to surgery	125	Observational study based on data from RCT	Plasma content of *n*−3 and *n*−6 fatty acids	n/a	Post-operative AF ≥ 5 min	None	Continuous ECG	Results suggestive of a U-curved relationship between quartiles of plasma *n*−3 PUFA and post-operative AF with the lowest incidence in the second quartile in a population with high background intake. Arachidonic acid was associated with a reduced risk of post-operative AF

AF, atrial fibrillation; n−3 PUFA, marine n−3 polyunsaturated fatty acids; RCT, randomized controlled trial; ECG, electrocardiogram; CABG, coronary artery bypass graft surgery.

time to onset of AF (HR 0.66, 95% CI 0.43–1.01). Length of stay in the intensive care unit (ICU) was significantly decreased in the *n*−3 PUFA group (ratio of means 0.71, 95% CI 0.56–0.90).

In opposition to these findings, two RCT showed no effect of *n*−3 PUFA on the incidence of post-operative AF. Thus, in a study by Saravanan et al. (2010a) of 108 patients scheduled for CABG and randomized to 1.7 g *n*−3 PUFA/day or placebo and followed for 5 days with continuous ECG, no difference in AF was found between groups. Compared to the above studies, the definition of AF was duration ≥30 s instead of >5 to >15 min, which explains a high rate of AF. The other study by Heidarsdottir et al. (2010) in Iceland with 168 patients treated with CABG and/or valve surgery and randomized to 2.2 g *n*−3 PUFA/day or placebo found no difference between groups. However, the background diet in this population was rich in cod liver oil and *n*−3 PUFA supplements, and the percentage of *n*−3 PUFA in plasma phospholipids was about three times higher than that reported in an Italian study (Abbatecola et al., 2009) which may be comparable to the study by Calo et al. (2005). For this reason, the high background intake of *n*−3 PUFA and a relatively small increase in the intervention group may explain why no effect of marine *n*−3 PUFA could be demonstrated.

In a study of the acute effects of *n*−3 PUFA, 102 patients undergoing CABG were randomized to intravenous infusion 100 mg/kg/day of fish oil or placebo (Heidt et al., 2009). Infusion was started at admission and continued until the end of follow-up at transfer from the ICU. Post-operative AF occurred in 15 patients (31%) in the control group but only in 9 (17%) in the *n*−3 PUFA group ($p < 0.05$). The *n*−3 PUFA-treated patients also had a significantly shorter stay in the ICU.

The available data seem fairly promising, but more studies are needed to establish whether *n*−3 PUFA supplementation is beneficial in patients undergoing heart surgery. Possible effects in this special setting of heart surgery with a high inflammatory response may not be directly comparative to other settings such as primary or secondary prevention. However, prevention of post-operative AF by a safe and well-tolerated treatment would be a compelling treatment option as this would reduce morbidity in these patients as well as the time spent in hospital. For future studies, a longer pre-operative treatment for at least 1 month may be considered to allow for a higher incorporation of *n*−3 PUFA into cardiac cell membranes.

n−3 PUFA AND AF PATIENTS WITH PACEMAKERS

In a study of AF patients with dual-chamber pacemakers, Biscione et al. (2005) conducted an open-label serial intervention with 1 g *n*−3 PUFA/day and observation by pacemaker interrogation before treatment, after 4 months of treatment, and after 4 months of withdrawal (**Table 4**). A significant reduction in number of episodes and burden of atrial tachyarrhythmia was found during *n*−3 PUFA treatment. These interesting findings, however, need to be confirmed in other studies.

n−3 PUFA AND RADIO FREQUENCY ABLATION

A lower risk of early recurrence (≤8 weeks) and procedural failure (>8 weeks) was reported in a nested case–control study from a cohort of patients treated with pulmonary vein antrum ablation

for AF (Patel et al., 2009; **Table 4**). Among the 1500 treated patients, patients consuming ≥665 mg of fish oil were matched with controls (129 in each group). Also, in an unblinded, randomized study on patients undergoing pulmonary vein isolation, patients randomized to 1.8 g *n*−3 PUFA/day had prolonged pulmonary venous and left atrial effective refractory periods and decreased susceptibility to initiation of AF from within the pulmonary veins (Kumar et al., 2011b). In comparison, the effects of *n*−3 PUFA on electrophysiologic parameters were studied in a single-blinded study of patients with supraventricular tachycardia but without AF or structural heart disease (Kumar et al., 2011a). The participants had a low fish intake and were randomized to 1.8 g *n*−3 PUFA/day for at least 1 month, and effects of *n*−3 PUFA treatment included lengthening of atrial refractory period, less inducible, and shorter duration of AF.

WHY ARE RESULTS ON MARINE *n*−3 PUFA AND AF INCONSISTENT?

One explanation for the mixed results of the effects of *n*−3 PUFA could be that the effect is not simply a linear dose–response but may be, e.g., a threshold effect between very low intake and normal/high intake or even a J- or U-shaped effect where very low and very high levels are detrimental, as suggested in a study on plasma content of *n*−3 PUFA and post-operative AF (Skuladottir et al., 2011). Also, as AF is a disease that may be caused by a wide range of different factors and in turn, *n*−3 PUFA also has been ascribed a long list of possible mechanisms of action on AF, the association of effect is likely to vary between populations according to background intake of *n*−3 PUFA, the overall health status of patients, and the type and duration of AF.

When comparing information from observational studies and intervention studies, one must keep in mind that there are important differences in the amounts of *n*−3 PUFA investigated in these two types of designs. Observational studies often compare persons with higher intakes against persons with very low intakes, whereas in interventional studies a high dose is usually investigated against a control group with an average intake that represents the whole study population. For this reason, for example if there is a threshold effect of *n*−3 PUFA between the lowest quartile of fish intake, this may not be tested appropriately in a standard RCT. Likewise, if there is an upper limit to the beneficial effect of *n*−3 PUFA, this could also be a problem in RCT where some persons are likely to have a high background intake of fish supplemented with high doses in capsules.

The concomitant treatment with other drugs that may potentially work by some of the same mechanisms as *n*−3 PUFA may also be relevant. Thus, in a recent study in patients with a history of myocardial infarction, low-dose supplementation with *n*−3 fatty acids was associated with a reduction in major cardiovascular events in patients not treated with statins, whereas in statin users no difference was found (Eussen et al., 2012). Medications that affect atrial remodeling by reducing atrial fibrosis and also reduce AF may include statins and inhibitors of the renin–angiotensin–aldosterone system (Nattel et al., 2008). It is possible that the potential effect of additional treatment with *n*−3 PUFA in these patients may be less.

A problem when studying AF is that while some patients are bothered by symptoms of AF and therefore more likely to be diagnosed, a large proportion are unaffected and only diagnosed by chance or not at all. Also, even symptomatic patients have asymptomatic episodes of AF. Thus, the proportions of symptomatic and asymptomatic patients vary between studies according to the background population, and recurrence rates during follow-up are increased depending on the completeness of heart rhythm monitoring during follow-up (e.g., continuous or ambulant ECG) which will have a major effect on the outcome and may explain the diverging results obtained.

KEY QUESTIONS FOR FURTHER RESEARCH

Based on the available studies in regard to AF after cardioversion or heart surgery, it seems reasonable to consider a pre-procedure treatment of at least 30 days for interventional studies examining the effect of dietary *n*−3 PUFA. Future studies are needed to clarify whether a relation between dietary intake of *n*−3 PUFA and AF is linear, U- or J-shaped or has a threshold above which no further effect is obtained. More knowledge on this has great implications both for comparing studies in populations with different background dietary intake and dose of exposure, and for establishing the optimal dosage. It is likely that some conditions will have a higher potential for an effect of *n*−3 PUFA, and identifying these conditions would be important for patient treatment. Experimental data suggest differences between the acute effects of intravenous infusion of *n*−3 PUFA and the chronic effects of dietary *n*−3 PUFA incorporated into cell membranes, and therefore studies should be interpreted according to the type of exposure. Finally, the endpoint in most studies is first episode of AF. Although this is a measurable endpoint, it may well be that if only a small proportion can achieve freedom from AF, patients with AF would still be interested in a treatment option that may reduce the number and severity of episodes and burden of time in AF, as exemplified by Biscione et al. (2005).

CONCLUSION

There is growing evidence for an effect of marine *n*−3 PUFA in prevention and treatment of AF. However, further studies are needed to establish which patients are more likely to benefit from *n*−3 PUFA, timing of treatment, and dosages.

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Polyunsaturated fatty acids in atrial fibrillation: looking for the proper candidates

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Atrial fibrillation (AF) is the most common sustained arrhythmia encountered in clinical practice with growing prevalence in developed countries. Several medical and interventional therapies, such as atrial specific drugs and pulmonary vein isolation, have demonstrated prevention of recurrences. However, their suboptimal long-term success and significant rate of secondary effects have led to intensive research in the last decade focused on novel alternative and supplemental therapies. One such candidate is polyunsaturated fatty acids (PUFAs). Because of their biological properties, safety, simplicity, and relatively cheap cost, there is a special clinical interest in omega-3 PUFAs as a possible antiarrhythmic agent. Obtained from diets rich in fish, they represent one of the current supplemental therapies. At the cellular level, an increasing body of evidence has shown that *n*-3 PUFAs exert a variety of effects on cardiac ion channels, membrane dynamic properties, inflammatory cascade, and other targets related to AF prevention. In this article, we review the current basic and clinical evidence pertinent to *n*-3 PUFAs in AF treatment and prevention. We also discuss controversial outcomes among clinical studies and propose specific subsets of AF patients who will benefit most from *n*-3 PUFAs.

Keywords: atrial fibrillation, omega-3 polyunsaturated fatty acids, remodeling, prevention, drug therapy

INTRODUCTION

Atrial Fibrillation (AF) is the most common sustained arrhythmia encountered in clinical practice (Kannel et al., 1998). In developed countries the arrhythmia is associated with doubling of mortality in both sexes and is one of the main causes of embolism (Wolf et al., 1991). Although AF already represents an important health care problem, its prevalence is estimated to increase in the next decade (Miyasaka et al., 2006). AF is classified by how long the patient has had the arrhythmia. AF that lasts less than 7 days is classified as paroxysmal. AF that lasts longer than 7 days, without a return to sinus rhythm, is designated as persistent. Persistent AF lasting for more than 1 year is termed long-standing AF as long as a rhythm control strategy is still pursued. However, when the rhythm control is no longer pursued the arrhythmia is designated as permanent AF (Camm et al., 2010). Current therapeutic options, either antiarrhythmic drugs (amiodarone, flecainide, vernakalant, dronedarone, etc.) or radiofrequency catheter-based procedures have limited efficacy and are not completely free of complications (Lafuente-Lafuente et al., 2006; Cappato et al., 2010). This is because as the arrhythmia perpetuates there is substantial atrial remodeling that promotes the maintenance of AF (Nattel et al., 2008).

Therefore, as the prevalence of AF increases, preventing its first episode or recurrences before it becomes permanent is a crucial component of treatment. Currently, therapies aimed at preventing recurrences (Healey et al., 2005; Lafuente-Lafuente et al., 2006; Singh et al., 2007) have not demonstrated an elimination of the arrhythmia burden or a wide safety profile. While in the last decade new multichannel profile and more atrial specific antiarrhythmic drugs have been developed, they are still not without substantial side effects and can be pro-arrhythmic (Kober et al., 2008). Therefore, novel therapeutic options are needed to improved AF management (Dobrev et al., 2012). An increasing amount of scientific evidence has suggested fish oil dietary supplement as a simple, safe, and relatively cheap adjunct to existing therapies (Mozaffarian et al., 2004; Calo et al., 2005; Nodari et al., 2011). Here, we aim to review the current knowledge regarding the role of polyunsaturated fatty acids (PUFAs) as a therapeutic used for the treatment and prevention of AF. Using a bench to the bedside approach, we will attempt to identify the appropriate AF candidates who will successfully respond to PUFAs.

BIOCHEMISTRY OF POLYUNSATURATED FATTY ACIDS

Fatty acids are essential constituents of the cell membrane. Categorized by the length of their chains they are classified as short (fewer than 6 carbon atoms), medium (6–12 carbon atoms), or long (greater than 12 carbon atoms). They are further classified as saturated or unsaturated. Saturated fatty acids have no double bonds between their carbon atoms allowing the molecule to become "saturated" with the maximal number of possible hydrogen atoms attaching. Unsaturated forms of fatty acids possess biophysical, structural, and dynamic properties essential for normal cellular function (Leaf et al., 2005). For Omega-3 (*n*-3 fatty acids), the terminal double bond is on C3 counting from the methyl end of the hydrocarbon chain. The more commonly encountered *n*-6 (or omega-6) PUFAs have the terminal double bond on C6. The

long hydrocarbon chain of the *n*-3/*n*-6 PUFAs, with multiple double bonds and with the first double bond occurring in the C3/C6 position result in complex and unique 3-dimensional configurations of PUFAs that are essential to their biological properties (Mozaffarian and Wu, 2011).

Fatty acids required for normal physiological functions that are produced by the body but obtained from food are called essential. Two of these are linoleic (*n*-6 PUFA) and α-linolenic (*n*-3 PUFA) acids. Once ingested, biochemical pathways can further metabolize essential fatty acids into long-chain more unsaturated derivatives. Linoleic acid can be converted into arachidonic acid and α-linolenic acid into eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively; Burdge et al., 2002). However, endogenous conversion is very limited in humans (8% assuming the best scenario), which makes tissue and circulating EPA and DHA levels primarily dependent on direct dietary consumption (Burdge, 2004). While plants are a good source of linoleic and α-linolenic acids, longer chain *n*-3 fatty acids are mainly obtained from fish (tuna, herring, mackerel, etc.). In humans, fish oil supplements result in progressively higher proportions of EPA, DHA, and total *n*-3 PUFAs in atrial phospholipids, along with a reciprocal lowering of long-chain *n*-6 PUFAs, predominantly arachidonic acid (Metcalf et al., 2007). Animal and human studies demonstrate a progressive incorporation of *n*-3 PUFAs in the myocardial cell membrane over a 30-day period (Owen et al., 2004; Metcalf et al., 2007). Certain factors such as age, diabetes, body mass index, etc. could affect the incorporation of *n*-3 PUFAs into the cell membrane (Sands et al., 2005). Therefore, the slow and gradual incorporation of *n*-3 PUFAs into the membrane phospholipids should be taken into consideration when assessing the beneficial effects of PUFAs supplemental therapy, since a delay in protection can be expected. This has been observed when prevention of total mortality and sudden cardiac death was examined after the initiation of *n*-3 PUFA supplementation (Marchioli et al., 2002). Similarly, diverging results on the rate of recurrences after a DC shock in patients with persistent AF may also be partially explained by differences in the duration of *n*-3 PUFA therapy before cardioversion (Bianconi et al., 2011; Nodari et al., 2011).

CELLULAR EFFECTS OF POLYUNSATURATED FATTY ACIDS

A large body of evidence supports the role of *n*-3 PUFAs in stabilizing the cardiomyocyte membrane and altering cardiac cells' electrophysiology (Leaf et al., 2003). Interestingly, some experiments have demonstrated that *n*-3 PUFAs exert their effects without strong ionic or covalent binding to specific targets in the cell membrane. It is currently accepted that their incorporation within the hydrophobic tail of the cardiomyocyte membrane phospholipids is sufficient to elicit electrophysiological changes, and even antiarrhythmic action (Kang and Leaf, 1994). However, direct interaction with proteins of certain cardiac ion channels such as Nav1.5 has also been suggested by introducing single amino acid mutations in the wild type protein that significantly reduced the expected potency of EPA to inhibit the fast sodium current (I_{Na}; Xiao et al., 2001).

The effects of PUFAs on cardiac ionic currents have been proposed as a major player in protection against AF. Acute effects of PUFAs on the biophysical properties of ion channels may substantially differ from those of chronically administered PUFAs that are gradually incorporated into the cell membrane (Den Ruijter et al., 2007). In human atrial cells, EPA and DHA acutely inhibit I_{Na} by shifting the potential of I_{Na} availability toward more negative voltages and increasing I_{Na} inactivation at resting states (Li et al., 2009). This effect on I_{Na} has also been observed in HEK 293 cells expressing human cardiac sodium channels and in neonatal rats (Xiao et al., 1995, 1998). In contrast with the acute effects, peak I_{Na} was unaffected by incorporated *n*-3 PUFAs in ventricular myocytes isolated from pigs and rats fed with a diet rich in fish oil (Leifert et al., 2000; Verkerk et al., 2006).

In addition to affecting I_{Na} the antiarrhythmic properties of PUFAs have also been attributed to their capability to modulate the L-type calcium channel ($I_{Ca\text{-}L}$; Xiao et al., 1997) and the human ether-a-go-go-related gene (HERG) channel, which mediates the repolarizing rapid delayed rectifier K^+ current (I_{Kr}; Guizy et al., 2005). Similar to the effects described on I_{Na}, no changes were observed on I_{Kr} density and calcium homeostasis (diastolic Ca^{2+} and Ca^{2+} transient amplitude) in pig ventricular myocytes with incorporated sarcolemmal *n*-3 PUFAs (Verkerk et al., 2006). In the same study and in line with the acute administration (Xiao et al., 2004), chronic administration of *n*-3 PUFAs resulted in reduced Na^+-Ca^{2+} exchange current (I_{NCX}). This reduction may explain the decreased propensity to develop delayed after depolarizations (Blaustein and Lederer, 1999). Moreover, changes in membrane phospholipids have been observed in rat atrial myocytes after a fish oil dietary supplement compared to saturated and monounsaturated diets. The fish oil dietary supplement was associated with calcium sparks of smaller area, and shorter duration in cells with a higher ratio of *n*-3 to *n*-6 PUFAs (Honen and Saint, 2002). This may contribute to prevent diastolic calcium release and decrease the propensity of delayed after depolarizations, which can initiate AF.

With regards to the other repolarizing potassium currents, in human atrial cells, EPA and DHA significantly inhibit the ultrarapid delayed rectifier K^+ current (I_{kur}) and the transient outward K^+ current (I_{to}; Li et al., 2009). The inhibition of I_{kur} is of special interest since represents an atrial specific current (Li et al., 1996), and its blockade might lead to AF termination (Blaauw et al., 2004). Although attractive, specific I_{kur}-blockade may not be effective to terminate persistent AF and certain AF models, in which I_{Kur} is decreased and increased inward rectifier K^+ currents may counterbalance the potential effect of blocking I_{Kur} in action potential duration (Van Wagoner et al., 1997; Pandit et al., 2011). Chronic treatment with EPA at low concentrations (1 μM) may increase I_{Kur} current. Conversely higher concentrations of DHA and EPA (30 μM; more physiological) decrease the expression of Kv1.5 protein channel (principal molecular component of I_{Kur}; Koshida et al., 2009).

In human atrial cells, no significant effects have been recorded in the main inward rectifier potassium current (I_{K1}) after exposing the cells to oleic acid (Crumb et al., 1999). The same results were obtained in ferret cardiomyocytes after acute administration of *n*-3 PUFAs (Xiao et al., 2002). Conversely, incorporation of *n*-3 PUFAs into the sarcolemma results in I_{K1} increase by ≈50%, which shortens the action potential duration, reduces delayed after

depolarizations and triggered activity (Verkerk et al., 2006; Den Ruijter et al., 2008). The slow delayed rectifier K^+ current (I_{Ks}) also shows significant increase after incorporation of *n*-3 PUFAs in the sarcolemma of ventricular myocytes (Verkerk et al., 2006). Acute effects on I_{Ks} differ depending on the type of *n*-3 PUFA. While DHA has shown to increase I_{Ks} magnitude, EPA does not exert any significant effect on I_{Ks} (Doolan et al., 2002).

Unsaturated free fatty acids such as oleic, linoleic, and arachidonic acids reversibly inhibited the ATP-dependent gating of native acetylcholine-sensitive K^+ current ($I_{K\text{-}Ach}$) in rat atrial cells (Kim and Pleumsamran, 2000). Free unsaturated fatty acids of the cardiac cell membrane seem to be crucial to keep the $I_{K\text{-}ACh}$ channel in the short-lived, single open state, and maintain low channel activity despite the presence of ATP in the cell. The role of $I_{K,Ach}$ in AF is well described in experimental models of AF. Activation of these channels causes hyperpolarization of the resting membrane potential and shortening of the action potential duration. Heterogeneous distribution of $I_{K,ACh}$ channels in the atria generate non-uniform distribution of refractory periods and make the atria prompt to AF (Moe and Abildskov, 1959; Sarmast et al., 2003).

In addition, *n*-3 PUFAs might affect stretch activated channels (SAC), which are non-specific ion channels activated in response to mechanical deformation of the membrane. In rat atrial myocytes, membrane compliance increases after acute addition of the *n*-3 PUFAs DHA and EPA (Jahangiri et al., 2000). Langendorff-perfused hearts of rabbits on a dietary fish oil supplement were more resistant to stretch-induced AF compared to hearts from controls (Ninio et al., 2005). This is similar to the results of earlier experiments (Bode et al., 2001) after blocking SAC by the tarantula venom peptide GsMtx-4. Consequently, it could be speculated that an increase in membrane fluidity could protect against stretch-induced vulnerability to AF. However, it remains unknown whether PUFAs modify the biophysical properties of SAC.

Polyunsaturated fatty acids may also act through alternative mechanisms derived from their effects on inflammation, endothelial function, atherosclerosis, etc. Through the activation of transcription factors such as peroxisome proliferator-activated receptors (PPARs) and nuclear factor kappa B (NFκB), *n*-3 PUFAs are able to regulate metabolism and other cell and tissue responses, such as inflammation. Healthy human volunteers who undertook a dietary fish oil supplement showed decreased production of tumor necrosis factor-α (TNFα), interleukine-1β (IL-1β), IL-6, and various growth factors by monocytes or mononuclear cells that were stimulated with bacterial endotoxin (Endres et al., 1989; Trebble et al., 2003). Changes in cell membrane composition after *n*-3 PUFA supplementation alter the production and potency of eicosanoid and eicosanoid-like mediators produced from the *n*-6 PUFA arachidonic acid (prostaglandins, thromboxanes, and leukotrienes). These have well-established roles in the regulation of inflammation and immunity (Calder, 2006).

Therefore, based on acute effects and incorporation of *n*-3 PUFAs into the cardiac cell membrane, PUFAs may decrease atrial heterogeneity and Ca^{+2}-induced triggered activity, both leading to lower risk of AF onset and recurrences. Altogether, cellular studies suggest that long-term *n*-3 PUFAs supplementation may act as an upstream therapy for substrate modification and membrane stabilization rather than a pure antiarrhythmic agent. Summarized acute and chronic *n*-3 PUFAs effects on cardiac ion currents are shown in **Table 1**.

Table 1 | Acute vs. chronic *n*-3 PUFAs effects or cardiac ion currents.

Author/s Year	Ion Current	PUFAs Effects		Cell type
		Acute	Chronic	
Li et al. (2009); Xiao et al. (1995, 1998)	I_{Na}	Decrease		Human atria, HEK 293, neonatal rat ventricular myocytes
Leifert et al. (2000); Verkerk et al. (2006)			Unaffected	Pig and rat ventricular myocytes
Li et al. (2009)	I_{Kur}	Decrease		Human atria
Koshida et al. (2009)			Decrease (30 μM)	Transfected green monkey kidney fibroblast cells and rat atrium
Li et al. (2009)	I_{to}	Decrease	Unknown	Human atria
Xiao et al. (1997)	$I_{Ca\text{-}L}$	Decrease		Rat ventricular myocytes
Verkerk et al. (2006)			Decrease	Pig ventricular myocytes
Xiao et al. (2004)	I_{NCX}	Decrease		HEK 293t
Verkerk et al. (2006)			Decrease	Pig ventricular myocytes
Guizy et al. (2005)	I_{Kr}	Decrease		Chinese hamster ovary cells expressing HERG
Verkerk et al. (2006)			Unaffected	Pig ventricular myocytes
Doolan et al. (2002)	I_{Ks}	Increase (DHA)		KvLQTI and hminK injected in Xenopus oocytes
Verkerk et al. (2006)			Increase	Pig ventricular myocytes
Xiao et al. (2002)	I_{Kl}	Unaffected		Adult ferret cardiomyocytes
Verkerk et al. (2006)			Increase	Pig ventricular myocytes
Kim and Pleumsamran (2000)	$I_{K\text{-}ACh}$	Decrease	Unknown	Rat atrium
	SAC	Unknown	Unknown	

SAC, stretch activated channels; DHA, docosahexaenoic acid.

EFFECTS OF POLYUNSATURATED FATTY ACIDS IN EXPERIMENTAL MODELS OF AF

Experimental models of AF show much less variability than human populations at large. This allows the identification of specific mechanisms or substrates suitable for potential treatment with PUFAs. Models mainly based on electrical remodeling, structural remodeling, or inflammatory-related models can provide valuable insights to understand the role of *n*-3 PUFAs in clinical AF.

Oral supplementation with *n*-3 PUFAs (DHA and EPA acids), commencing 2 weeks before tachypacing onset and continuing through the fast pacing period (7 days), did not significantly affect AF duration and atrial refractory period compared to sham-operated controls in a dog model of AF where the ventricular rate was controlled by atrioventricular block and ventricular demand pacing. Further, dogs that underwent ventricular tachypacing with the same regimen of *n*-3 PUFA supplementation showed decreased congestive heart failure-related atrial fibrosis and attenuated AF promotion induced by ventricular tachypacing (Sakabe et al., 2007). The authors found significantly decreased expression of phosphorylated mitogen-activated protein (MAP) kinases, which are particularly important in causing tissue fibrosis in both heart failure animals and AF patients (Goette et al., 2000; Petrich and Wang, 2004). In a dog model of AF with simultaneous fast atrial and ventricular pacing, *n*-3 PUFA supplementation resulted in less conduction time heterogeneity in the left atrium, and prevented pacing-induced increase in collagen turnover and collagen deposition in atrial appendages. PUFAs reduced both AF inducibility and duration of inducible AF (Laurent et al., 2008). Echocardiographic assessment of mechanical remodeling in those animals showed a similar decrease in left atrial-emptying function in treated animals and controls (Laurent et al., 2008).

However, there are discrepancies about the effects of PUFAs in the literature that appear to be model dependent. For example, acute administration of PUFAs prevented atrial electrical remodeling by significantly reducing the shortening of atrial effective refractory period caused by several hours of fast atrial pacing in dogs (Da Cunha et al., 2007). This acute effect was not observed in the same animal model of PUFAs treatment under long-term fast atrial pacing (Sakabe et al., 2007). Interestingly, both dietary supplements and acute administration of *n*-3 PUFAs prevented vagally induced AF in dogs (Sarrazin et al., 2007). Experimental results in vagally induced AF correlate with the relevant role of PUFAs in the cardiac cell membrane to modulate $I_{K,ACh}$ as described above.

The antiarrhythmic effects of PUFAs have been observed in non-tachypaced models of AF as well. Accumulating evidence indicates that inflammatory pathways are of significance in AF. Although some evidence suggests that inflammation might be a causative agent for AF (Sata et al., 2004), a substantial body of evidence supports that AF and inflammatory pathways have a bidirectional relationship (Friedrichs et al., 2011). After cardiac surgery, leukocytosis and pro-inflammatory cytokines have been directly related to the incidence of post-operative AF. As the cytokines raise, the risk of post-operative AF concomitantly increases (Ishida et al., 2006). *n*-3 PUFAs show anti-inflammatory effects that may prevent AF episodes related to a highly inflammatory environment. Experimentally, in a canine model of open-chest sterile pericarditis, oral PUFAs supplement for 4 weeks before the operation and 2 days afterward resulted in less AF inducibility and maintenance than in a control group under regular feeding. Before the operation, there were no significant differences in conduction time, atrial effective refractory period (AERP; defined as the longest S_1–S_2 coupling interval that fails to depolarize the atria) and inflammatory markers between PUFAs group and controls. Two days after surgery, C-reactive protein (CRP), IL-6, and TNF-α levels were significantly lower in the PUFAs group. PUFAs supplementation also resulted in longer AERP and shorter intra-atrial conduction time after surgery (Zhang et al., 2011).

MECHANISMS UNDERLYING AF AND THEIR LINK TO CELLULAR AND EXPERIMENTAL PUFAs EFFECTS

Although the mechanisms underlying AF are not completely understood, the arrhythmia is believed to be reentrant. There is increasing evidence supporting the role of a unique or small number of functional reentrant sources (rotors) maintaining the arrhythmia (David Filgueiras Rama and José Jalife, 2011). This is largely because of the elucidation of the molecular mechanisms of reentry. Theoretically, it has been known that shortening of the action potential duration and increasing excitability can facilitate reentry (Pandit et al., 2005). However, it was not until the last decade that the role of inward rectifier K^+ currents, such as $I_{K,ACh}$ or I_{K1}, and their ability to increase reentrant frequency and facilitate AF became a well-established molecular mechanism responsible for AF. For a complete review on the role of inward rectifiers (see Ehrlich, 2008; Jalife, 2011). While $I_{K,ACh}$ may have a preferential role in paroxysmal AF and explain left-to-right differences in rates of activation (Voigt et al., 2010), the current seems to decrease in persistent AF. However, ionic remodeling leads to an increase in I_{K1} and constitutive active $I_{K,ACh}$ (Dobrev et al., 2001, 2005; Makary et al., 2011). Conversely, as AF becomes persistent extensive data show decrease in I_{to}, I_{Kur}, I_{Na}, and the L-type Ca^{+2} current (Van Wagoner et al., 1997, 1999; Dobrev et al., 2001; Sossalla et al., 2010).

While reentry seems to perpetuate the arrhythmia, Ca^{2+}-dependent triggered activity may initiate AF. While the spontaneous release of Ca^{+2} and triggered activity implicate abnormal sarcoplasmic reticulum (SR) Ca^{+2} release as a trigger, the frequency of triggered activity, and spontaneous Ca^{+2} release are much slower than the typical AF activation rate (<1 vs. 6–9 Hz, respectively; Atienza et al., 2006, 2009; Voigt et al., 2012). As a result, it is unlikely they are the mechanism maintaining the arrhythmia. This idea is further supported by AF models like stretch-induced AF, in which a more depolarized resting membrane potential and the activation of SAC enable the generation of triggered activity. Even in the presence of a high rate of focal activity reentry was required to sustain AF (Filgueiras-Rama et al., 2012).

However, it is important to note that as the arrhythmia persists, electrical remodeling and functional changes in subcellular structures lead to higher susceptibility to Ca^{2+}-dependent triggered activity. Ryanodine (RyR2) dysfunction and SR Ca^{2+} leak may contribute to further paroxysms and persistence of AF. Under certain conditions of excitability, anatomic and functional obstacles may interfere with propagation of regular or Ca^{2+} dependent waves, which may cause the formation of self-sustained vortices.

Concomitantly, larger inward Na^+-Ca^{2+}-exchange current (I_{NCX}) for a given SR Ca^{2+} release further increase the likelihood of delayed after depolarizations and triggered activity (Voigt et al., 2012).

In addition to electrical remodeling, structural changes can facilitate the long-term maintenance of AF (Nattel et al., 2008). Structural changes include atrial dilatation and an increase in atrial fibrosis. This is a consistent finding in AF models associated with congestive heart failure (Morillo et al., 1995; Li et al., 1999). Extracellular matrix dysregulation and atrial fibrosis increases atrial conduction heterogeneity and plays an important role in stabilizing reentry and making larger areas of the atria suitable for harboring reentry. Different inter related signaling pathways appear to be involved in the development of atrial fibrosis. The most prominent pathways studied are the renin-angiotensin system (RAS), transforming growth factor-β1 (TGF-β1), and the inflammation/oxidative stress pathways (Lin and Pan, 2008). Furthermore, elevated inflammatory mediators varied according to the different sub-types of the arrhythmia. There is a graded increase in N-terminal pro-brain natriuretic peptide (NTpBNP) and TNF-α with the duration and type of AF (permanent > persistent > paroxysmal). Patients with lone AF (no overt structural heart disease) are less likely to have elevated concentrations of biomarkers (IL-10, TNF-α, and NTpBNP; Li et al., 2010). Baseline levels of biomarkers and further decrease after cardioversion may also be used to predict sinus rhythm maintenance during the following year in patients with lone AF (Leftheriotis et al., 2009). However, structural remodeling does not occur in all AF models. Predominant electrical remodeling with minimal or no changes in atrial fibrosis are present in tachypacing induced AF models even after long periods of fast pacing, as long as tachycardiomyopathy is not present (Ausma et al., 1997).

Synthesizing all this together, the role of *n*-3 PUFAs in terminating AF by modulation of cardiac ion channels seems unlikely based on the limited effects observed with chronic PUFAs supplements. Moreover, once AF is initiated, the increase in I_{K1} after PUFAs supplement may facilitate reentry (Verkerk et al., 2006). However, *n*-3 PUFAs have also been shown to prevent AF, and it is speculated that an increase in membrane fluidity and I_{K1} may protect against stretch-induced triggered activity and AF initiation. Similarly, a decrease in abnormal Ca^{+2} release may also prevent focal triggered activity initiating AF (Honen and Saint, 2002). The crucial role of PUFAs to control ATP-induced increase in K_{ACh} channel activity may also be important in persistent AF, in which $I_{K,ACh}$ is constitutively active. Additionally, it has been proposed that the PUFAs antiarrhythmic effects lie in the ability of *n*-3 PUFAs to modify the atrial substrate that perpetuates the arrhythmia. Thus, PUFAs have the capability to attenuate the inflammatory cascade and adverse remodeling occurring in response to mechanical stress (Sakabe et al., 2007; Laurent et al., 2008).

CLINICAL IMPLICATIONS OF PUFAs IN AF POPULATION

The AF population represents a large source of variability. Therefore, specific AF subsets could benefit more than others from using *n*-3 PUFA dietary supplementation. It is not surprising there are different responses to PUFAs depending on the type of AF (lone, paroxysmal, persistent, and permanent). Further, the evaluation of the efficacy of PUFAs is convoluted by concomitant therapies, degree of inflammatory biomarkers, and time-course of the supplementary dietary therapy before outcomes analysis. Based on the experimental studies we have discussed so far, we speculate that PUFAs are not predominantly antiarrhythmic ion channel blockers. Rather, they play an important role to prevent AF onset in disease states with strong inflammatory and structural remodeling components. Results from different trials highlight the potential role of *n*-3 PUFAs in preventing AF onset and recurrences mainly from persistent AF populations, which supports their role as upstream therapy.

The study by Calo et al. (2005) in patients who underwent coronary artery bypass graft (CABG) surgery showed that *n*-3 PUFA supplementation (EPA/DHA ratio 1:2) at least 5 days before surgery reduced the incidence of post-operative AF by ≈50%. Further, it was associated with a shorter hospital stay (Calo et al., 2005). Similar results were observed in a randomized trial with the same type of surgery and same PUFAs regimen (Sorice et al., 2011). Conversely, another randomized clinical trial in patients who underwent CABG surgery did not show statistical differences between *n*-3 PUFAs and placebo groups (Saravanan et al., 2010). This discrepancy may be explained by different designs of the studies. The EPA/DHA supplementation ratio was 1:2 in Calò's study and 1.2:1 in the study by Saravanan et al. (2010). Considering that DHA may have greater impact on AF prevention (Virtanen et al., 2009), the results may be somehow affected by the study design. It also should be noted that Calò's study included AF episodes lasting >5 min compared to >30 s in Saravanan's. This resulted in a much higher AF incidence, without a clear clinical significance of such short episodes. Finally, the use of concomitant β-blockers and statins was much lower in the study by Calò (85 and 98% compared with 57 and 56%). Optimization of these agents could have decreased the beneficial effects of *n*-3 PUFAs.

Two other randomized trials in patients who underwent cardiac surgery either CABG and/or valvular replacement did not show any beneficial effect of *n*-3 PUFAs (EPA/DHA ratio 1.2/1.4:1) in preventing post-operative AF compared to controls (Heidarsdottir et al., 2010; Farquharson et al., 2011). The study by Heidarsdottir et al. (2010) was carried out in an Icelandic population with approximately 80% of participants taking cod liver oil or other fish oils, which resulted in very small changes of plasma *n*-3 PUFAs concentrations after the regimen. In the second study by Farquharson et al. (2011) the results showed a trend to toward a decrease in incidence of AF in the *n*-3 PUFAs group. However the sample size was underestimated for the AF rates observed in the study, which resulted in no statistically significant differences. The discussed clinical studies evaluating new-onset AF following cardiac surgery are summarized in **Table 2**.

Beyond post-operative AF, over a 12-year follow-up period regular consumption of *n*-3 PUFAs (tuna and other broiled or baked fish ≥5 times per week) showed ≈30% lower incidence of AF among subjects older than 65 years after adjustment for other risk factors (Mozaffarian et al., 2004). Although those observational results were encouraging, subsequent randomized trials brought up controversial results. The study by Nodari et al. (2011), in patients with persistent AF and at least one relapse after cardioversion, showed significant decrease in AF recurrences after

Table 2 | Clinical trials in new-onset AF following cardiac surgery.

Author year	Age (years)	Design	Study population	PUFAs dosage	No of patients	Duration	Results
Calo et al. (2005)	65.6 ± 8.5	Open label/ randomized	Pre/post-CABG, SR	EPA/DHA 1:2	160	At least 5 days before CBAG until discharge	Reduced post-CABG surgical AF and shorter hospitalization
Saravanan et al. (2010)	66 (58–73)	Double blind/ randomized	Pre/post-CABG, SR	EPA/DHA 1.2:1	108	At least 5 days before CBAG until discharge/5 days	No reduction in AF after CABG surgery
Heidarsdottir et al. (2010)	67 (43–82)	Double blind/ randomized	Pre/post-cardiac surgery, SR	EPA/DHA 1.2:1	168	5–7 days before CABG and/or valvular repair until discharge/14 days	No reduction in AF after cardiac surgery
Farquharson et al. (2011)	64 ± 11	Double blind/ randomized	Pre/post-cardiac surgery, SR	EPA/DHA 1.4:1	194	3 weeks before CABG and/or valve replacement until discharge/6 days	Trend to decrease in post-surgical AF. Decreased length of stay in the ICU
Sorice et al. (2011)	63 ± 10	Open label/ randomized	Pre/post-cardiac surgery, SR	EPA/DHA 1:2	201	At least 5 days before CBAG until discharge	Decrease in "on-pump" CABG surgical AF

AF, atrial fibrillation; CABG, coronary artery bypass graft surgery; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ICU, intensive care unit; PUFA, omega-3 polyunsaturated fatty acids; SR, sinus rhythm.

Table 3 | Main clinical trials in non-postoperative AF onset and recurrent AF.

Author year	Age (years)	Design	Study population	PUFAs Dosage	No of patients	Duration	Results
Mozaffarian et al. (2004)	72(65–100)	Observational prospective	Population-based	Tuna, baked fish	4815	12 years	Lower incidence of AF with fish intake ≥1 time per week
Virtanen et al. (2009)	52.8 ± 5.3	Observational prospective	Population-based men. SR	Fish intake	2174	17.7 years	High serum levels of *n*-3 PUFAs decrease hospital diagnosis of AF
Kowey et al. (2010)	60.5 ± 12.8	Double blind/ Randomized	Symptomatic paroxysmal/persistent AF, SR	EPA/DHA 1.2:1	663	24 weeks after enrollment	No reduction in recurrent AF
Nodari et al. (2011)	69.5 ± 7	Double blind/ Randomized	Recurrent persistent AF >1 month + ACE-Is + Amiodarone	EPA/DHA 1.2:1	199	1 year	Decrease in persistent AF recurrences post-cardioversion
Bianconi et al. (2011)	69.2 ± 7.9	Double blind/ Randomized	Persistent AF >1 month. Mainly "lone AF"	EPA/DHA 1.2:1	204	6 months after enrollment	No reduction in persistent AF recurrences post-cardioversion
Kumar et al. (2012)	61 ± 13	Open label/ Randomized	Persistent AF >1 month ± ACE-Is/Amiodarone/Sotalol	EPA/DHA1.3:1	178	1 year	Decrease in persistent AF recurrences post-cardioversion

ACE-Is, angiotensin-converting enzyme inhibitors; AF, atrial fibrillation; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, omega-3 polyunsaturated fatty acids; SR, sinus rhythm.

cardioversion and *n*-3 PUFA supplementation (EPA/DHA ratio 1.2:2). More recently, in a similar AF population, the study by Kumar et al. (2012) showed that fish oil supplementation resulted in a sixfold prolongation in the median time to AF recurrence compared to controls. Conversely, two other randomized trials by Kowey et al. (2010); Bianconi et al. (2011) did not show any beneficial effect of *n*-3 PUFAs in preventing AF recurrences in persistent AF patients after cardioversion or in sinus rhythm patients with previously documented AF, respectively. Again, several methodological factors may partly explain these discordant findings. Nodari's and Kumar's studies included patients taking *n*-3 PUFA supplementation for at least 4 weeks before cardioversion, with a history of at least one previous cardioversion. Nodari's series also enrolled a population with high prevalence (≈90%) of structural heart disease. Most patients in Bianconi's study (≈60%) were experiencing their first episode of AF and only 25% of patients had a previous cardioversion. In addition, before cardioversion, the length of *n*-3 PUFAs therapy was shorter than the time required for incorporation of PUFAs in the cell membrane (≈28 days). This may explain why the majority of recurrences occurred very early in follow-up (2–3 weeks), before the expected biological effects of *n*-3 PUFAs. In addition, in both studies with negative results the presence of structural heart disease was significantly lower than in Nodari's study, which may represent a key factor in understanding the potential role of PUFAs in preventing structural changes preferentially in patients with significant

structural heart disease, similarly to the additional clinical benefits of spironolactone therapy in patients with AF and structural heart disease (Williams et al., 2011). The clinical studies evaluating AF incidence and recurrences are summarized in **Table 3**.

FUTURE DIRECTIONS

Data from animal studies seem to support the role of *n*-3 PUFAs as a therapeutic option in AF. AF models with intense structural remodeling due to heart failure and ventricular tachypacing, post open-chest surgery, and vagally induced AF have shown to benefit from *n*-3 PUFA supplementation. These effects are also supported by some of the effects of *n*-3 PUFAs at the cellular level. However, more mechanistic insights are necessary to further understand the specific pathways that make *n*-3 PUFAs an effective adjunctive therapy to prevent AF. Arguably, this is because structural remodeling of the atria and acute inflammation may affect AF susceptibility in a different manner based on the inflammatory pathways involved. In fact, recent data have shown that some of those inflammatory markers affect the activity of certain cardiac ion channels, as well as the interaction between cardiomyocytes and fibroblasts (Ottaviano and Yee, 2011; Ramos-Mondragon et al., 2011). As a result new experimental data is needed to understand how *n*-3 PUFAs influence the release of inflammatory markers and how those markers influence AF.

Changes in lipid composition of the sarcolemma seem to modulate some cardiac ion currents such as $I_{K,ACh}$ and at least indirectly affect membrane susceptibility to stretch (Kim and Pleumsamran, 2000; Ninio et al., 2005). Specific changes in *n*-3 PUFAs composition in the lipid membrane of cardiomyocytes after dietary supplementation are currently unknown and need to be addressed. It is also necessary to study the specific *n*-3 PUFAs effects on SAC beyond the increase in membrane compliance.

Finally, DHA and EPA seem to exert different effects at the cellular level and clinical outcomes (Virtanen et al., 2009). New clinical trials based on mechanistic effects described in experimental studies will be necessary to avoid confounding factors that appear to be present in the current clinical trials.

CONCLUSION

Biochemical and biophysical properties of *n*-3 PUFAs give rise to a variety of effects at the cellular and organ levels. They include increasing cardiomyocyte membrane stability, modulation of the biophysical properties of ion channels/cellular substructures, and significant effects on the inflammatory/fibrosis signaling pathways. In light of experimental and clinical studies, the latter may be especially important in preventing AF (post-operative or clinical recurrences). Consequently, dietary supplementation of *n*-3 PUFAs may be considerably more beneficial in patients with pronounced structural heart disease and atrial remodeling, high levels of inflammatory biomarkers, and low baseline levels of circulating PUFAs. New ongoing clinical trials, mainly focus on AF recurrences and post-operative AF, will hopefully help to pinpoint the specific subset of AF population that will benefit most from *n*-3 PUFA supplementation.

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Effects of *n*−3 polyunsaturated fatty acids on cardiac ion channels

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Dietary *n*−3 polyunsaturated fatty acids (PUFAs) have been reported to exhibit antiarrhythmic properties, and these effects have been attributed to their capability to modulate ion channels. In the present review, we will focus on the effects of PUFAs on a cardiac sodium channel ($Na_v1.5$) and two potassium channels involved in cardiac atrial and ventricular repolarization (K_v) ($K_v1.5$ and $K_v11.1$). *n*−3 PUFAs of marine (docosahexaenoic, DHA and eicosapentaenoic acid, EPA) and plant origin (alpha-linolenic acid, ALA) block $K_v1.5$ and $K_v11.1$ channels at physiological concentrations. Moreover, DHA and EPA decrease the expression levels of $K_v1.5$, whereas ALA does not. DHA and EPA also decrease the magnitude of the currents elicited by the activation of $Na_v1.5$ and calcium channels. These effects on sodium and calcium channels should theoretically shorten the cardiac action potential duration (APD), whereas the blocking actions of *n*−3 PUFAs on K_v channels would be expected to produce a lengthening of cardiac action potential. Indeed, the effects of *n*−3 PUFAs on the cardiac APD and, therefore, on cardiac arrhythmias vary depending on the method of application, the animal model, and the underlying cardiac pathology.

Keywords: *n*−3 PUFAs, atrial fibrillation, arrhythmias, heart failure

INTRODUCTION

Omega-3 (*n*−3) polyunsaturated fatty acids (PUFAs) are essential nutrients that must be acquired from the diet and that are required for normal development and cellular function. *n*−3 PUFAs are derived from two sources: (1) ALA (18:3 *n*−3), which is found in vegetable oils (such as flaxseed, canola, and soybean oils) and walnuts, and (2) EPA (20:5 *n*−3) and DHA (22:6 *n*−3), which are found in oily fish, fish oil, and seafood (**Figure 1**). After the Industrial Revolution, the dramatic increase in the *n*−6-to-*n*−3 ratio in the diets of the populations of Western countries has, at least in part, contributed to the rise in cardiovascular disease (CVD; De Caterina et al., 2003; Leaf et al., 2003). Sinclair et al. described the rarity of CVD in Greenland Eskimos, who consumed a diet rich in *n*−3 PUFAs (whale, seal, and fish; Sinclair, 1953, 1956). Since that time, a large amount of evidence from cellular, animal studies (Billman et al., 1997, 1999; Leifert et al., 2000), and from clinical trial outcomes (Burr et al., 1989; GISSI-Prevenzione Investigators, 1999; Tanaka et al., 2008; Tavazzi et al., 2008) has suggested that an increased intake of fish oil fatty acids has favorable effects on cardiovascular health. Analyses of these trials have concluded that these beneficial effects mainly occur through the prevention of sudden cardiac death (SCD), which is often preceded by ventricular arrhythmias, indicating that *n*−3 PUFAs are antiarrhythmic (GISSI-Prevenzione Investigators, 1999; Marchioli et al., 2002). However, not all studies have demonstrated the cardioprotective effects on CVD of PUFA consumption. Proarrhythmic actions have been described for *n*−3 PUFAs in animal models during acute regional myocardial ischemia (Coronel et al., 2007). Moreover, the recent Alpha OMEGA and OMEGA randomized trials, involving patients who had suffered a myocardial infarction, did not show any improvement in the clinical results following *n*−3 PUFAs supplementation (Kromhout et al., 2010; Rauch et al., 2010), and a deleterious effect due to an increased risk of cardiac death was reported in men with stable angina (without myocardial infarction) who were advised to eat fish (Burr et al., 2003), and in patients with implantable cardioverter defibrillators (ICDs; Raitt et al., 2005). These differences could be explained by the fact that a diet rich in fish oil could be pro- or antiarrhythmic depending on the underlying arrhythmogenic mechanism.

In any case, the mechanism underlying the anti- or pro-arrhythmic effect after *n*−3 supplementation is thought to be related to the modulation of the cardiac ion channels involved in the genesis and/or maintenance of cardiac action potentials (APs). *n*−3 PUFAs inhibit the fast sodium current (I_{Na}), ultrafast activating delayed outward potassium current (I_{Kur}), transient outward potassium current (I_{to}), rapidly activating delayed rectifying outward potassium current (I_{Kr}) L-type calcium inward current (I_{Ca}), and Na^+-Ca^{2+} exchange current (I_{NCX}), and enhanced slowly activating delayed rectifying outward potassium current (I_{Ks}) and inward rectifying potassium current (I_{K1}; Honoré et al., 1994; Xiao et al., 1995, 1997; Doolan et al., 2002; Jude et al., 2003; Guizy et al., 2005, 2008; Verkerk et al., 2006; Dujardin et al., 2008). Because the configuration and duration of the cardiac AP are highly relevant for arrhythmogenesis, in the present review, we summarize the acute and chronic effects of *n*−3 PUFAs on the I_{Na}, I_{Kur}, and I_{Kr} (**Figure 2**) and we will relate these effects with the pro- and antiarrhythmic effects on different animal models of arrhythmia, as well as to the results observed in the clinical trials.

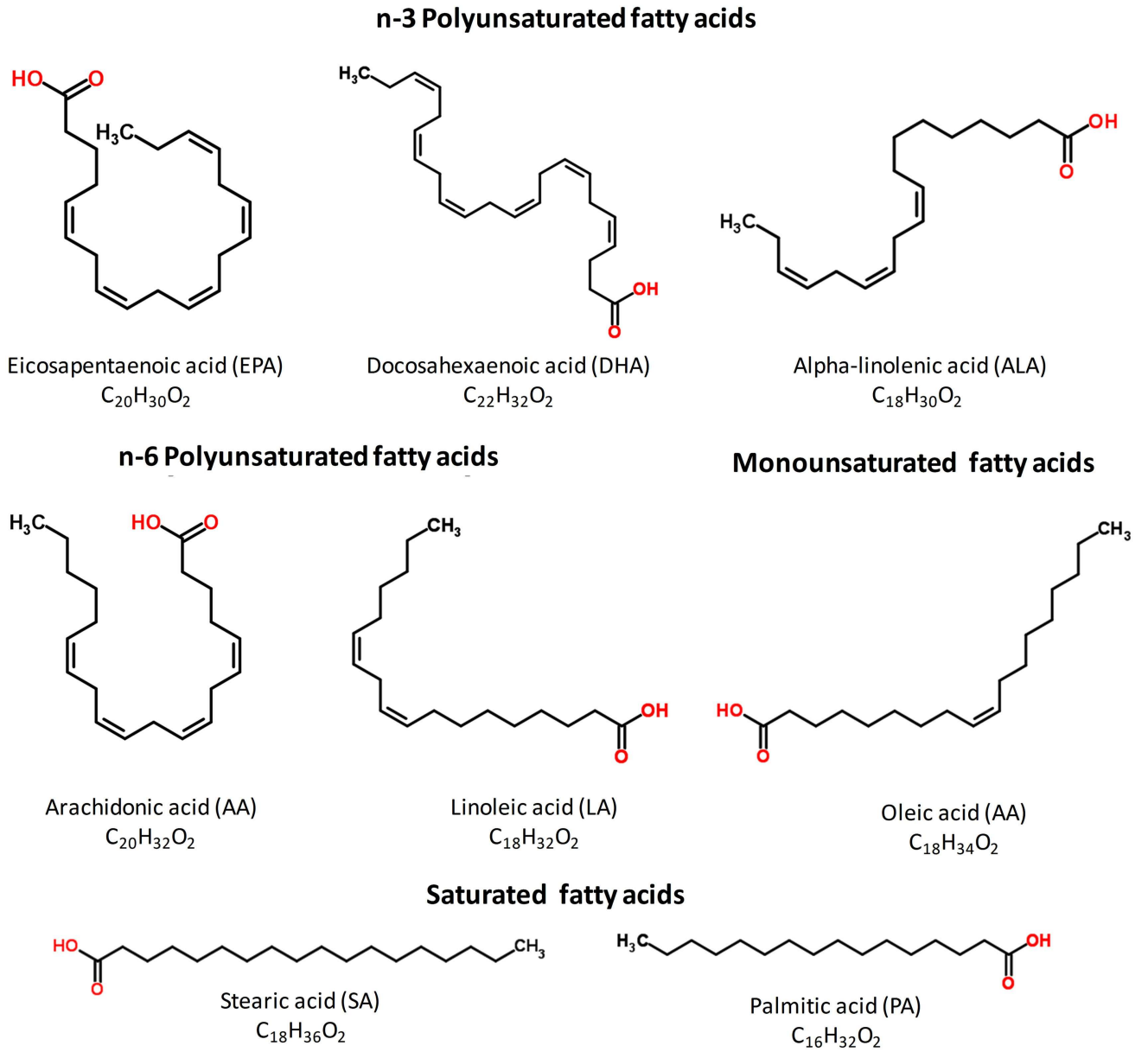

FIGURE 1 | Structure of the main *n*−3 (EPA, DHA, and ALA), *n*−6 (AA and LA), monounsaturated (OA), and saturated (SA and PA) PUFAs present in the human diet. Taken from the free ChemSpider Database (http://www.chemspider.com/).

EFFECTS OF *n*−3 PUFAs ON SODIUM CHANNELS

The fast inward cardiac sodium current is responsible for the genesis of the AP (phase 0) in most cells of excitable tissues and plays a pivotal role in impulse conduction. Thus, the ability of agents to modulate I_{Na} may constitute an important factor to prevent, terminate, or exacerbate cardiac arrhythmias (Echt et al., 1991).

In 1995, it was reported that EPA reduced membrane electrical excitability of heart myocytes, increased the threshold for the genesis of the cardiac AP and hyperpolarized the resting membrane potential (Kang et al., 1995). As it was expected, when the effects of EPA were studied on the fast sodium current in isolated neonatal rat cardiac myocytes (Xiao et al., 1995) or in the current generated after activation of cloned human $Na_v1.5$ expressed in a mammalian cell line (Xiao et al., 1998), an inhibition of the I_{Na} occurred. These effects on sodium channels were concentration-, time-, and voltage-dependent with an IC_{50} of 4.8 and 0.5 μM respectively. These IC_{50} values are within the *n*−3 PUFAs physiological plasma range in the human being (Burtis et al., 1999). EPA shifted the inactivation curve toward more negative potentials by ∼20 mV whereas the activation kinetics of the channel was unaffected. EPA slightly hyperpolarized the resting membrane potential (likely due to an enhancement of I_{K1}) and made more positive the threshold for the activation of $Na_v1.5$ channel. In addition, the transition from the rested to the inactivated state

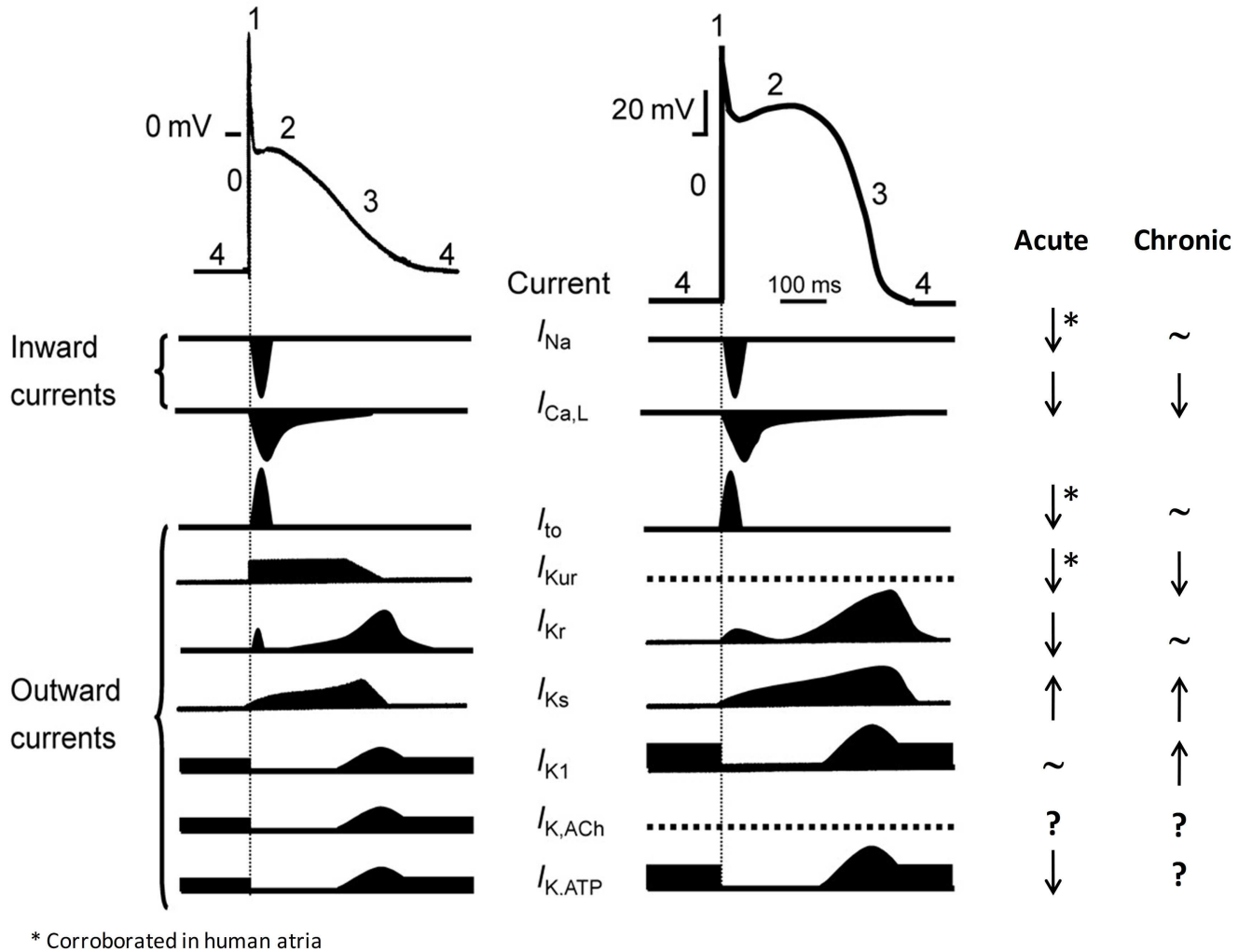

FIGURE 2 | Cardiac AP, ionic currents involved in its genesis and maintenance, and the effects of *n*−3 PUFAs on each current under acute and chronic conditions. ↑, Increase; ↓, decrease; or ∼, no changes in the current amplitude. *Studied in human atria. ?, Not tested. With permission from Ravens and Cerbai (2008).

was markedly accelerated in the presence of EPA but the recovery from inactivation was only slightly prolonged (Xiao et al., 1998; Dujardin et al., 2008). This effect could account for the greater relative refractory period-action potential duration (APD) ratio. Other *n*−3 (DHA and ALA), *n*−6 PUFAs (AA), and monounsaturated fatty acid (oleic acid, OA) reduced I_{Na} being the *n*−3 PUFAs the more potent ones.

In adult rat cardiomyocytes, sodium current was also inhibited by EPA, DHA, and ALA, DHA being the most potent *n*−3 PUFA blocking sodium channels (IC_{50} of 6 μM), followed by EPA (IC_{50} of 16 μM) and ALA (IC_{50} of 27 μM). Similarly to that observed in I_{Na} recorded in neonatal cardiomyocytes; DHA, EPA, and ALA shifted the voltage dependence of inactivation toward more negative potentials (∼20–23 mV). However, and in contrast to the effects on the sodium channels of neonatal cardiomyocytes, these three PUFAs shifted the voltage dependence of activation toward more positive potentials (∼8–12 mV; Leifert et al., 1999). In contrast with the acute effects, peak I_{Na} was unaffected by incorporated *n*−3 PUFAs in ventricular myocytes isolated from pigs and rats that were fed a diet rich in fish oil (Leifert et al., 2000; Verkerk et al., 2006). In both studies voltage dependence of activation remained unaltered, whereas a shift (<8 mV) in the inactivation toward more negative potentials was observed.

It is not clear how PUFAs modulate the activity of ion channels, if they modify the current through indirect effects on the lipid bilayer or if they directly interact with the channel protein. The dominant view is that *n*−3 PUFAs directly interact with the protein of the ion channel. A very useful technique used to establish an interacting site is to introduce single directed point mutations in the wild type protein and to determine whether the substitution modifies the expected action of the ligand. Due to the striking

similarities between the *n*−3 PUFAs effects and those induced by local anesthetics, the effects of *n*−3 PUFAs were analyzed on $Na_v1.5$ channels carrying point amino acid mutations that have been shown to modify the action of local anesthetics (Xiao et al., 2001). Interestingly, the substitution of an asparagine by a lysine at position 406 (N406K) at the segment 6 of the domain 1 of $Na_v1.5$ channel significantly reduced the potency of EPA to inhibit I_{Na}, mimicking bupivacaine effects and suggesting a direct interaction with this protein (Nau et al., 2000; Xiao et al., 2001). Also, the effects of *n*−3 PUFAs on the sodium current generated by the activation of $Na_v1.5$ channels expressed in HEK293 cells were modified by the cotransfection with accessory β subunits (Xiao et al., 2000).

On the basis of these results, sodium channel blockade produced by *n*−3 PUFAs is responsible, at least in part, for the antiarrhythmic or proarrhythmic effects observed in clinical trials. In the clinics, class I antiarrhythmic drugs, such as flecainide, are useful in the treatment of atrial fibrillation (Fuster et al., 2011). Thus, we can speculate that the sodium current inhibition properties of *n*−3 PUFAs can account for the beneficial effects observed in clinical trials focused on atrial fibrillation (Calo et al., 2005). Conversely, blockade of the cardiac sodium channel can be deleterious under certain pathological conditions in which arrhythmias are based on reentrant activity, such as during acute myocardial ischemia. Sodium channel blockade by acute administration of *n*−3 PUFAs, likely facilitates reentry by slowing conduction velocity and incorporated *n*−3 PUFAs shorten APD and effective refractory period (ERP), because these conditions favor a reentrant circuit to arise. In fact, an increase in the risk of cardiac events has been reported in patients with acute ischemia (e.g., angina pectoris) and in patients with histories of sustained ventricular tachycardia or ventricular fibrillation, as well as arrhythmias triggered by reentry (Burr et al., 2003; Brouwer et al., 2009).

EFFECTS OF *n*−3 PUFAs ON REPOLARIZING CURRENTS

The ultrarapid activated outward potassium current is the native counterpart to $K_v1.5$ channels in human atria and contribute to the repolarization process of the atrial AP (Fedida et al., 1993; Snyders et al., 1993). Since the early nineties, it is known that $K_v1.5$ current is inhibited by the acute exposition to *n*−3 (DHA), and *n*−6 PUFAs (AA), but not by monounsaturated (OA) and saturated fatty acids (palmitic acid, PA and stearic acid, SA) in stably expressing $K_v1.5$ mammalian cells (Honoré et al., 1994). The inhibition was time- and concentration-dependent and occurred at physiological concentrations (IC_{50} for AA and DHA ∼20–30 μM) only when these PUFAs were applied from the external side of the membrane. Both *n*−6 and *n*−3 fatty acid shifted the activation curve toward more negative potentials ∼16 mV and accelerated the activation process. All these results are indicative of an open channel mechanism of block. The same characteristic block was induced by DHA and AA on the I_{Kur} current in mouse neonatal cardiomyocytes, in rat embryonic ventricular cells (Honoré et al., 1994) and, more interestingly, in human atrial myocytes (IC_{50} for EPA and DHA 17.5 and 4.3 μM, respectively; Li et al., 2009).

Guizy et al. (2008) compared acute versus chronic effects of the plant derived *n*−3 PUFA ALA on the inhibition of $K_v1.5$ channels. The acute ALA blocking properties resembles to those previously described by Honoré et al. (1994) for DHA and AA. However, in the case of ALA, the $K_v1.5$ inhibition was also voltage-dependent. The chronic effects on the channel function and on its protein expression levels were assessed by incubating the cells with ALA 10 μM for 24 and 48 h. The effects of ALA on the $K_v1.5$ current were similar to those obtained when the cells were acutely exposed to ALA. EPA and DHA, but not ALA, reduced the steady-state levels of $K_v1.5$ protein in a concentration-dependent manner at concentrations higher than 30 μM. However, it has also described that chronic treatment with EPA at low concentrations (∼1 μM) stabilizes $K_v1.5$ channel protein in the endoplasmic reticulum and Golgi apparatus thereby enhancing the $K_v1.5$ channel current on the cell membrane (Koshida et al., 2009).

As mentioned above, $K_v1.5$ inhibition was observed when they were added to the external side of the membrane. This fact strongly suggests an interaction between *n*−3 PUFAs and ion channel, unlike the non-specific lipid-protein interactions proposed for marine *n*−3 PUFAs (Leaf and Xiao, 2001). This hypothesis was tested by measuring changes in the fluorescence anisotropy of the lipophilic dye PA-DPH on the membrane of Ltk^- cells in the presence of ALA or DHA. The PA-DPH molecule has an anion polar head and a lipid hydrocarbon chain that resembles membrane lipids. It is incorporated and anchored into the bilayer of the plasmalemma and its motion reflects the wobbling of its lipidic components. It was found that DHA but not ALA, modifies the PA-DPH motion in the Ltk^- cell membrane. From these experiments it was concluded that the apparent viscosity and the order of the membrane rather than the mobility of the bilayer components are not affected by ALA, but increased in cells incubated with DHA (Leifert et al., 1999; Guizy et al., 2008). These results do not permit us to exclude an effect of DHA on the biophysics of the lipid bilayer, besides its direct effects on ion channels. In fact, it has also been reported that *n*−3 PUFAs are able to change the composition of membrane microdomains (Basiouni et al., 2012; Turk and Chapkin, 2012).

The ultra rapid activating delayed rectifier potassium current I_{Kur}, together with I_{to}, repolarizes the cardiomyocyte membrane in human atria during the AP (Wang et al., 1993) but does not play a role in the ventricle even though its mRNA and protein are also present in that tissue (Mays et al., 1995). Thus, inhibition of I_{Kur} prolongs atrial but not ventricular APD and refractoriness. This finding indicates that I_{Kur} blockers can be useful in the treatment of supraventricular arrhythmias such as AF without the risk of ventricular pro-arrhythmia (Tamargo et al., 2004). Some of the cardioprotective effects attributed to *n*−3 PUFAs may be mediated by the inhibition of $K_v1.5$ current. This could be particularly relevant on the treatment of AF. This arrhythmia is associated with rapid shortening of APD and ERP. In fact, recent epidemiological studies points out an important role of *n*−3 PUFAs in the prevention of AF after coronary by-pass surgery (Calo et al., 2005; Mariscalco et al., 2010).

While atrial repolarization is mostly carried out by I_{to} and I_{Kur}, ventricular repolarization is developed by the slowly activating delayed rectifier (I_{Ks}) and the rapidly activating delayed rectifier (I_{Kr}) currents. The I_{Kr} is the native counterpart of $K_v11.1$ channels present in the human myocardium (Sanguinetti and Jurkiewicz, 1991; Sanguinetti et al., 1995) and its activity determines the

duration of the QT interval of the electrocardiogram (ECG) and therefore the refractory period (Li et al., 1996).

In 2002 it was demonstrated, for the first time, the inhibitory effects of DHA on I_K current (composed by I_{Ks} and I_{Kr}) in ferret myocytes. The extracellular application of DHA (10 μM) inhibited I_K on atrial and ventricular ferret myocytes with an IC_{50} of ∼20 μM. K_v11.1 blockade was concentration- but not voltage-dependent. Other PUFAs like the *n*−3 ALA and the *n*−6 linoleic acid also decreases the I_K magnitude whereas monounsaturated (OA) and saturated fatty acids (SA) did not (Xiao et al., 2002).

The same *n*−3 PUFA concentration (10 μM) was tested in another work to characterize the effects of DHA and AA on K_v11.1 human cloned channels (Guizy et al., 2005). Both, AA and DHA reduced the K_v11.1 current at the end of long depolarizing pulses and, to a greater extent, at the peak of the deactivation tail current, DHA being more potent. Also, K_v11.1 block was voltage-, time-, and use-dependent. AA and DHA: (1) shifted the activation curve toward more negative membrane potentials (−5 and −11 mV, respectively) and shifted the inactivation curve toward more positive potentials (∼12 mV); (2) accelerated the activation and the deactivation kinetics; (3) at high rate frequencies, the K_v11.1 peak current was exponentially reduced; and (4) did not modify the onset, the inactivation kinetics nor the recovery process. All these results suggest that AA and DHA preferentially bind to the open state of the channel (Guizy et al., 2005).

The effects of incorporated *n*−3 PUFAs were also evaluated on pig ventricular myocytes fed with a diet rich in *n*−3 PUFAs for 8 weeks. In this study, no changes on the amplitude and the properties of I_{Kr} were noticed, thus suggesting different effects of *n*−3 PUFAs after acute exposition than after feeding animals (i.e., chronic conditions; Verkerk et al., 2006).

n−3 PUFAs, like most antiarrhythmic drugs, modulate sodium, potassium, and calcium channels, as well as α- and β-adrenoceptors (Honoré et al., 1994; Xiao et al., 1995, 1997; Hondeghem et al., 2001; Jude et al., 2003; Guizy et al., 2005). EPA and DHA block sodium, calcium, and potassium currents with the exception of I_{Ks} and I_{K1} (Xiao et al., 1995, 1997; Doolan et al., 2002; Verkerk et al., 2006). Since *n*−3 PUFAs inhibit I_{Na}, I_{Ca}, and enhance I_{Ks} (effects that would shorten the cardiac action potential), but also inhibit I_{to}, I_{Kur}, and I_{Kr} (that should produce a lengthening of the APD), the result should be a modest effect on the time of repolarization. Besides these effects on the APD, it should be stated that its inhibitory effects on sodium, calcium, and several potassium channels (K_v4, and K_v11.1 channels) would result in a lengthening of the refractory period and a decrease of cardiac excitability, thus contributing to its antiarrhythmic effects.

The clinical consequences of the effects produced by *n*−3 PUFAs on K_v11.1 channels are likely dependent on the underlying pathology; but we cannot state any conclusion from the data available in the literature.

EFFECTS OF *n*−3 PUFAs ON ARRHYTHMIAS ANIMAL MODELS

In addition to the studies concerning the role of *n*−3 PUFAs on ion channels, several cellular, and animal models of induced arrhythmia have been created to better understand the mechanism by which *n*−3 PUFAs protect or exacerbate certain arrhythmias (Rosen et al., 1991; Members of the Sicilian Gambit, 2001). Traditional antiarrhythmic drug trials have yielded disappointing results, probably due to the inclusion of heterogeneous patient populations with different arrhythmogenic substrate. This led to the international cardiology community to focus their interest on the design of new rational approaches to study the effects of antiarrhythmic drugs based on mechanism- and disease-specific fashion. So, during the last years, the pro- or antiarrhythmic effects of *n*−3 PUFAs have been studied on different animal models with distinct underlying mechanisms of arrhythmia (triggered activity or reentry).

ANIMAL MODELS BASED ON ARRHYTHMIAS PRODUCED BY TRIGGERED ACTIVITY

The effects of EPA and DHA upon APD, triangulation, reverse use dependence, instability and dispersion (TRIaD) were studied in female rabbit fed with food enriched with *n*−3 PUFA (Dujardin et al., 2008). In this study, *n*−3 PUFAs significantly prolonged APD and ERP without triangulation. This apparent discrepancy with another studies (Verkerk et al., 2006; Den Ruijter et al., 2010) can be related to the fact that this study was performed in female rabbit hearts, whose repolarization is known to be slower and is modulated in a different manner (Gaborit et al., 2010; Lowe et al., 2012). This prolongation of APD resulted in a significant prolongation of the ERP. At baseline, *n*−3 PUFAs pre-treatment had no effects on TRIaD and on ectopic activity compared with control hearts. By exposing the rabbit heart to increasing dofetilide concentrations (1–100 nM) torsades de pointes (TdP, polymorphic ventricular arrhythmia due to triggered activity) was induced. It was studied whether the *n*−3 PUFA pre-treatment could prevent the proarrhythmic actions of dofetilide by decreasing TRIaD, and by suppressing the appearance of TdP. In control hearts, dofetilide-induced TdP in five of the six rabbit hearts, but none in the hearts from the *n*−3 PUFAs group. Hence, dofetilide-induced TRIaD was attenuated by pre-treatment with dietary supplements of *n*−3 PUFAs (Dujardin et al., 2008). Thus, a diet rich in *n*−3 PUFAs reduce the incidence of early afterdepolarizations (EADs) that could be also benefit in the treatment of long QT syndrome and other arrhythmias developed by triggered activity (e.g., EADs; Den Ruijter et al., 2006; Dujardin et al., 2008).

It has been described the beneficial effects of *n*−3 PUFAs in arrhythmias associated to heart failure. These beneficial effects have been reported when *n*−3 PUFAs are administrated acutely or as a diet supplementation. The acute administration of fish oil in cardiac myocytes from rabbits with volume- and pressure-overload-induced heart failure and of patients with end-stage heart failure inhibited triggered arrhythmias by lowering intracellular calcium and decreasing the response to noradrenaline (Den Ruijter et al., 2008). In this study, it is also reported a shortening of the cardiac APD induced by *n*−3 PUFAs. Recently, in the same animal model the authors demonstrate that supplementation of the diet with *n*−3 PUFAs reduces hypertrophy. They also describe a shortening of the cardiac action potential due to an increase in the magnitude of I_{to} and I_{K1}, a decrease of the Ca^{2+} transients and of the incidence of delayed afterdepolarizations. Thus, *n*−3 PUFAs prevented electric remodeling associated with heart

failure, leading to a reduced susceptibility of arrhythmias (Den Ruijter et al., 2012).

ANIMAL MODELS BASED ON REENTRANT ARRHYTHMIAS

Dietary *n*−3 fatty acids decreased the incidence of fatal heart disease relative to placebo (GISSI-Prevenzione Investigators, 1999). However, *n*−3 PUFAs tended to increase the risk of SCD in patients with angina pectoris (without myocardial infarction; Burr et al., 2003). Coronel et al. (2007) demonstrated in a pig ischemia model (produced by occluding the left anterior descending artery) that the supplementation of the diet with *n*−3 PUFAs during 8 weeks, did not modified ERP or the conduction velocity (neither longitudinal or transversal), although increased the incidence of arrhythmias. This study reports that those animals fed with *n*−3 PUFAs enriched diet show a reduced excitability, likely due to their effects on sodium channels, the main determinant of the substrate of ischemia-induced reentrant arrhythmias.

NEW INSIGHTS OF THE BENEFICIAL PUFAs PROPERTIES: PUFAs METABOLITES EFFECTS

In addition, genetic, pharmacologic, and biochemical studies are providing increasing mechanistically relevant explanations for the salutatory actions of EPA and DHA. *n*−3 and *n*−6 PUFAs are metabolized to lipoxins, resolvins, maresins (Serhan et al., 2008; Bannenberg and Serhan, 2010). These EPA- and DHA-derived lipid mediators (collectively termed Specific Pro-resolving Mediators, SPMs) are active, as anti-inflammatory agents, at very low concentrations (picomolar to nanomolar range), and are degraded locally by specific inactivating enzymes, characteristic for the bioactivity of autacoids. These SPMs can also slow the progression of diabetic onset, CVD, and aging-associated pathologies through the regulation of innate and adaptive immune responses. It has been described that lipoxins modulate CRAC channels after binding to the lipoxin receptor (Li et al., 2008). The effects of SPMs in the progression of several CVDs, involving inflammation (i.e., heart failure, diabetes, coronary heart disease) can be associated to the beneficial observed effects with *n*−3 PUFAs in the clinical trials. However, the cardiac electrophysiological effects of these SPMs are unknown at the present time. Thus, further studies focused on the analysis of their effects on cardiac ion channels and arrhythmias are necessary to better understanding the pro- or antiarrhythmic effects of *n*−3 PUFAs.

CONCLUDING REMARKS

n−3 PUFAs have diverse effects (increasing and decreasing) on cardiac ion currents. These differences appeared to depend on whether the *n*−3 PUFAs were applied to the cells or incorporated in the membranes modifying its composition and, thus, the effects on cell signaling and, therefore, in ion channel function. However, we cannot rule out PUFAs direct effects on ion channels, since once incorporated, *n*−3 PUFAs can also be released and there will arise an equilibrium between incorporated and circulating. *n*−3 PUFAs modulate cardiac ion currents, leading to a shortening of the cardiac APD. This effect can be beneficial or harmful, depending on the underlying pathology. Thus, during acute ischemia, in which the duration of the cardiac APD is already shortened (Behrens et al., 1997), a further decrease should be proarrhythmic. However, the APD shortening should be beneficial in preventing those arrhythmias caused by triggered activity. These differences can account, at least partially, with discrepancies observed in the clinical and epidemiological studies. Future studies analyzing the electrophysiological effects on cardiac ion channels and different models of arrhythmia of the *n*−3 metabolites are needed to better understanding the action of *n*−3 PUFAs.

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Are the anti-arrhythmic effects of omega-3 fatty acids due to modulation of myocardial calcium handling?

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Both animal and clinical studies have demonstrated that omega-3 fatty acids have anti-arrhythmic properties. It has been suggested that these anti-arrhythmic effects are due to modulation of the activity of various myocardial calcium handling proteins such as ryanodine receptor (RyR), L-type calcium current and sodium/calcium exchanger. In this article, we review all the data available on the effects of omega-3 fatty acids on ventricular myocardial calcium handling. In addition we highlight some unanswered questions and discuss possible therapeutic benefits of omega-3 fatty acids.

Keywords: omega-3, fatty acids, anti-arrhythmics, calcium, fish oils

INTRODUCTION

Omega-3 poly-unsaturated fatty acids (PUFA) have generated considerable interest as well as controversy regarding their role as anti-arrhythmic agents. A number of observational studies have shown that consumption of fish leads to a reduction in incidence of sudden deaths (Burr et al., 1989; Siscovick et al., 1995). The GISSI–Prevenzione trial, an open-label design trial found that 1 g/day of n-3 PUFA [(containing 289 mg of Eicosapentaenoic acid (EPA) and 577 mg Docosahexaenoic acid (DHA)] led to a 20% Relative Risk Reduction (RRR, 95% CI 6–23%) in total mortality after only 3 months of treatment without a significant change in myocardial infarction or stroke (1999). This suggested that the beneficial effects of PUFA are mainly related to an anti-arrhythmic action that prevents life-threatening arrhythmias. This generated a great deal of interest in the potential of PUFA as anti-arrhythmic agents and 3 prominent randomized, double blind, placebo controlled trials have been conducted (Leaf et al., 2005; Raitt et al., 2005; Brouwer et al., 2006). These trials analyzed the effects of PUFA on time to appropriate therapy for ventricular arrhythmias in ICD patients. They failed to demonstrate a convincing anti-arrhythmic effect and ignited the debate on whether PUFA have anti-arrhythmic properties. It has been suggested that the lack of efficacy of PUFA in patients with ICDs is due to the fact that this population contains patients with various arrhythmia mechanisms while the main anti-arrhythmic actions of PUFA are due to modulation of calcium handling leading to prevention of delayed after-depolarization (DAD) and triggered activity (TA) (Den Ruijter and Coronel, 2009). PUFA have also been demonstrated to diversely modulate a variety of cardiac ion channels (inhibit sodium, calcium and potassium currents other than I_{KS} and I_{K1}) as reviewed in detail in (Moreno et al., 2012) and hence the electrophysiological effects depend on the underlying cardiac pathology, setting of the arrhythmia and the method of application of PUFA. In this article we review the experimental evidence supporting the notion that the anti-arrhythmic actions of PUFA are due to modulation of myocardial calcium handling as well as prevention of DADs and TA. Whilst a variety of studies have also failed to reach a consensus on the effects of PUFA on the atrial myocardium, this review shall only focus on the effects of PUFA on calcium handling in the ventricular myocardium. To introduce the subject, we rapidly summarize normal myocardial calcium handling and the alteration in calcium handling that lead to DADs and TA.

Abbreviations: PUFA, Poly-Unsaturated Fatty Acids; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; ICD, Internal cardioverter-defibrillator; DAD, delayed afer-depolarization; TA, triggered activity; AP, action potential; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; SERCA, Sarcoplasmic reticulum Ca ATPase; ATP, adenosine triphosphate; NCX, Na^+/Ca^{2+} exchanger; SCR, spontaneous calcium release; VT, ventricular tachycardia; EAD, early after-depolarization; VF, ventricular fibrillation.

MYOCARDIAL CALCIUM HANDLING

In cardiac muscle, cytosolic calcium levels control the level of activation of the contractile proteins, the myofilaments and therefore the onset duration and intensity of contraction. The electrical activation with the spreading of the action potential (AP) throughout the heart initiates contraction by causing a transient increase in cytosolic calcium concentration, the systolic calcium transient (Bers, 2002). The AP generates the systolic calcium transient via a process called calcium-induced calcium release. During this process, the influx of a small amount of calcium via the sarcolemmal L-type calcium channels that are activated by the AP,

activates the sarcoplasmic reticulum (SR) calcium release channel, the ryanodine receptor (RyR), and triggers the release of calcium from the SR. Relaxation occurs after 100–200 ms when calcium gradually decays back to diastolic levels. This decline in cytosolic calcium concentration is due to termination of calcium release from the SR (inactivation of RyR) and rapid removal of calcium from the cytosol. Two systems are responsible for calcium removal from the SR—the sarcoplasmic reticulum calcium ATPase (SERCA) that uses ATP as energy to pump calcium back into the SR and the Na^+/Ca^{2+} exchanger (NCX) that exchanges the efflux of one Ca^{2+} (2 positive charges) with the influx of three Na^+ (3 positive charges). The activation of NCX leads to a net influx of a positive charge and therefore a net inward current. The above process is shown in **Figure 1A**.

MODULATION OF CA HANDLING

The amplitude of the calcium transient determines the level of activation of the myofilaments and therefore the intensity of contraction and it is finely modulated. The two main factors that determine the amount of calcium released from the SR and the amplitude of the calcium transient are: the amplitude of the L-type calcium current (Trafford et al., 2001) and the calcium

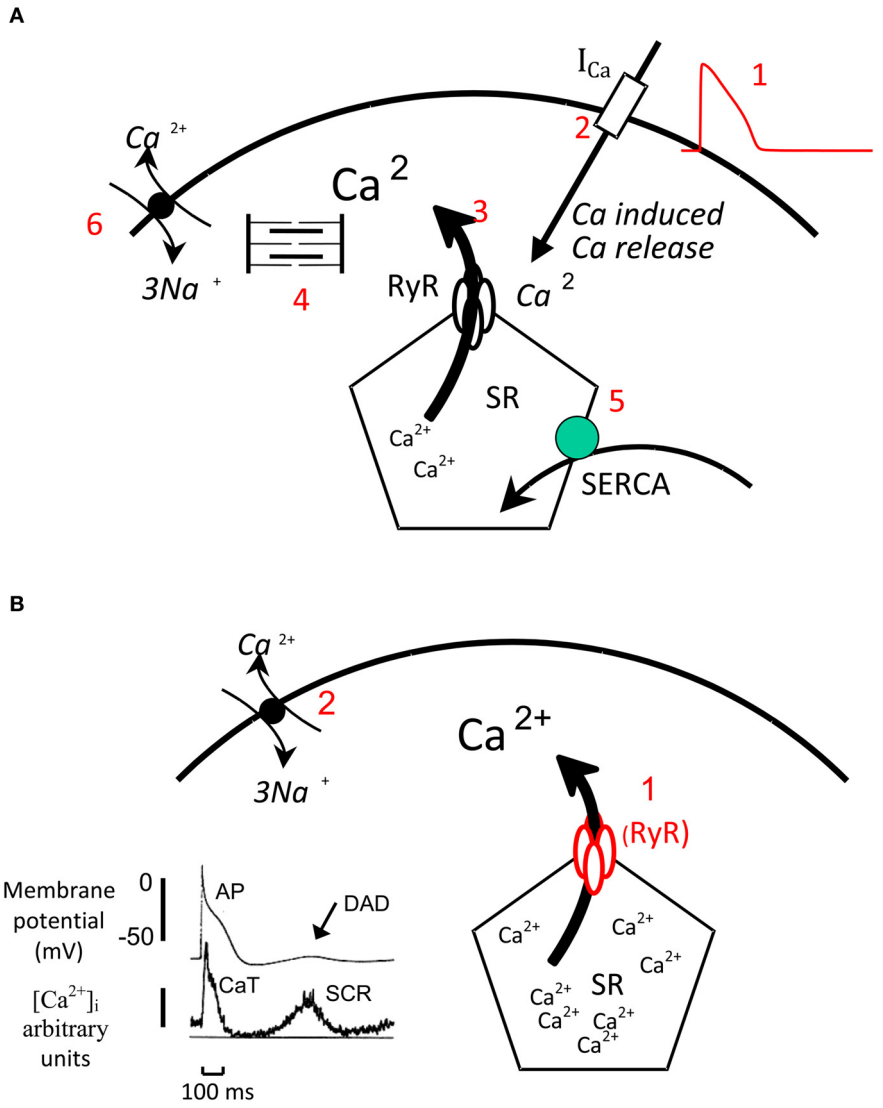

FIGURE 1 | Myocardial Calcium handling. (A) Normal Ca handling: The AP (1) activates the L-type calcium channels, the influx of a small amount of calcium via these channels (2) activates the RyR and triggers the release of a greater amount of calcium from the SR in to the cytosol (3). Calcium activates the myofilaments that generate contraction. During relaxation calcium is rapidly removed from the cytosol by SERCA (5) that pumps calcium back into the SR and by NCX that couples the efflux of 1 Ca^{2+} (two positive charges) to the influx of 3 Na^+ (three positive charges) and generates an inward current. **(B)** Generation of SCR and DADs: When intra SR calcium concentration is very high the SR can release calcium (1) independently from an AP this process is called spontaneous calcium release (SCR). This calcium activates the NCX (2) that generates an inward current and produces a delayed after—depolarization (DAD). The record on the left shows simultaneous recording of membrane potential and cytosolic calcium levels. The AP is triggers the calcium transient (CaT) while during diastole a SCR triggers a DAD.

concentration in the SR (Shannon et al., 2000). The SR Ca concentration can be increased by stimulation of SERCA activity and prolongation of the duration of the AP and decreased by stimulation of NCX activity.

In vivo the main modulator of the calcium transient amplitude is β adrenergic stimulation that via cAMP-mediated activation of protein kinase A stimulates both the L-type calcium channel and SERCA. This leads to an increase in L-type calcium current and SR Ca content and therefore a substantial increase in calcium transient amplitude (Hussain and Orchard, 1997).

ARRHYTHMIAS RELATED TO Ca HANDLING

Release of calcium from the SR can also occur independently from an AP during diastole (Venetucci et al., 2008). This process is called spontaneous calcium release (SCR) and occurs when the SR calcium concentration reaches a crucial threshold level (Diaz et al., 1997). In addition recently Belevych et al. (2012) elegantly demonstrated that soon after a systolic calcium transient the RyRs enter a refractory state and calcium wave occur only once the RyR have recovered from this refractory state even if the SR threshold is reached. Therefore the two conditions necessary for the generation of SCR are: (1) Increased SR Ca content up to the SR threshold for SCR (2) Recovery of RyR from refractory state. SCR activates the NCX that generates an inward current and a DAD. When a DAD reaches the threshold for activation of the Na channels, it initiates an AP which in turn causes TA and arrhythmias. The process of SCR and generation of DADs is shown in **Figure 1B**. SCR, DAD, and TA are responsible for the onset of arrhythmias in various clinical settings including digitalis-induced arrhythmias, some forms of heart failure and catecholaminergic polymorphic VT (a genetic arrhythmia syndrome caused by mutations of the RyR and CASQ2 genes). The scheme illustrated above suggests that there are two main therapeutic strategies that can be used to prevent the onset of calcium handling related arrhythmias: prevention of the onset of an SCR and prevention of triggering of an AP by a DAD. In view of the fact that the generation of DAD through SCR has been shown to be dependent on threshold SR calcium content (Diaz et al., 1997; Trafford et al., 2000; Jiang et al., 2005), the first strategy can be achieved by preventing the SR calcium content from reaching the threshold for SCR either by decreasing SR calcium content (Venetucci et al., 2007; Llach et al., 2011) or by raising SR threshold for SCR via inhibition of RyR (Venetucci et al., 2006; Maxwell et al., 2012). The second strategy can be achieved via inhibition of sodium channels that reduces the number of sodium channels ready to be activated when a DAD occurs and therefore reduces the likelihood that the DAD will initiate and AP and cause TA. Whilst this strategy can be beneficial in preventing arrhythmias caused by TA such as in heart failure, it could also be pro-arrhythmic during acute ischaemia by facilitating re-entry. In the following section, we will illustrate the experimental evidence that suggests that PUFA prevent SCR, DADs and TA via some of the mechanisms described.

EFFECTS OF PUFA ON MYOCARDIAL CALCIUM HANDLING

The effects of PUFA on calcium handling by cardiac myocytes have been extensively studied and an interesting picture has emerged. The effects vary depending on the modality of administration; two different modalities of application have been used: (1) application of free unesterified PUFA in external bath solution, (2) chronic diet supplementation that leads to incorporation in cardiac membrane as esters. To simplify, we will describe these effects separately.

EFFECTS OF FREE PUFA ON MYOCARDIAL CALCIUM HANDLING (ALSO SUMMARISED IN TABLE 1)

Application of free PUFA has profound effects on calcium handling. Macleod et al studied the effects of free PUFA on sodium and calcium currents and AP (Macleod et al., 1998). Both in rat as well as guinea pig isolated cardiac myocytes, free PUFA produced a dose-dependent reduction in sodium and calcium currents. The effects on AP duration were different in the two species. In rat ventricular myocytes, concentrations of EPA or DHA up to 7.5 μM caused AP prolongation. At concentrations above 10 μM AP shortening was observed. In guinea-pigs however AP shortening was observed at lower concentrations such as around 5 μM. In a second paper the same authors (Rodrigo et al., 1999) also demonstrated that PUFA at the same concentrations inhibit RyR. The inhibition of calcium current and the RyR and the shortening of the AP produce marked reduction in calcium transient amplitude and cell shortening. Stephen O'Neill's group studied in detail the effects of PUFA on Ca handling in rat ventricular myocytes (Negretti et al., 2000; O'Neill et al., 2002). They first studied the effects of EPA and DHA on RyR and confirmed that PUFA inhibit RyR and also increase the SR threshold for SCR (Negretti et al., 2000). In the same paper, they also demonstrated that free PUFA have no effects on NCX. In a follow-on paper (Szentandrássy et al., 2007), they also analyzed the effects of EPA on L-type calcium current, SERCA function and calcium transient. As previously described by Rodrigo et al. EPA reduces calcium current amplitude (Rodrigo et al., 1999). Interestingly EPA also increased SERCA activity by promoting phosphorylation of phospholamban. The characterization of the calcium transient demonstrated that 5 μM EPA reduced calcium transient if the myocytes were stimulated using voltage-clamp and increased it if the cell was stimulated using current-clamp. During current-clamp the cells are allowed to express their AP and the application of EPA (via inhibition of the transient outward current) produces a substantial prolongation of the AP. This increases SR calcium content substantially and therefore increases calcium transient amplitude despite inhibition of the L-type calcium current. During voltage clamp the cell is not allowed to express its AP. The membrane potential is controlled and the cell is stimulated using a 100 ms membrane potential step. The application of EPA does not produce any change in membrane potential and therefore does not produce a significant increase in SR Ca content. EPA still reduces calcium current amplitude and this reduction cause a significant reduction in calcium transient amplitude.

Free PUFA have also been shown to reduce the response to adrenergic stimulation. In 1995 Kang and Leaf (1995) demonstrated that the application of free PUFA to spontaneously beating rat neonatal myocytes attenuated the response to isoprenaline. PUFA reduced the increase in beating frequency produced by isoprenaline and prevented the onset of contracture. More recently,

Table 1 | Summarising effects of free PUFA on myocardial calcium handling.

Authors	Species studied	Findings
Macleod et al., 1998	Rat and guinea pig isolated cardiac myocytes	1. Dose dependant reduction in sodium and calcium currents 2. Rat - or DHA up to 7.5 μM caused AP prolongation Guinea pig—AP shortening was observed already at lower concentrations such as around 5 μM
Rodrigo et al., 1999	Rat and guinea pig isolated cardiac myocytes	Inhibition of calcium current and the RyR and the shortening of the AP produce marked reduction in calcium transient amplitude and cell shortening
Negretti et al., 2000 and O'Neill et al., 2002	Rat ventricular myocytes	1. PUFA inhibit RyR and also increase the SR threshold for SCR 2. Free PUFA have no effects on NCX
Szentandrássy et al., 2007	Rat ventricular myocytes	1. EPA reduces calcium current amplitude 2. EPA also increased SERCA activity by promoting phosphorylation of phospholamban 3. The characterization of the calcium transient demonstrated that 5 μM EPA reduced calcium transient if the myocytes were stimulated using voltage-clamp and increased it if the cell was stimulated using current-clamp
EFFECTS ON ADRENERGIC STIMULATION		
Kang and Leaf, 1995	Rat neonatal myocytes	Application of free PUFA to spontaneously beating rat neonatal myocytes attenuated the response to isoprenaline, reduced the increase in beating frequency produced by isoprenaline and prevented the onset of contracture
Den Ruijter et al., 2008	Rabbit and human myocytes	In rabbit myocytes, free PUFA reduced amplitude of the calcium transient and attenuated the increase in calcium transient amplitude produced by noradrenaline. In addition, in the presence of PUFA noradrenaline did not prolong the AP and failed to induce EADs and DADs. Similar effects were detected during experiments on human cardiac myocytes derived from severe congestive cardiac failure hearts explanted during cardiac transplantation.
Szentandrássy et al., 2007	Rat ventricular myocytes	PUFA reduce cAMP levels but directly stimulate PKA

Den Ruijter et al. (2008) characterized the effects of PUFA on the adrenergic response in rabbit and human myocytes (shown in **Figure 2**). In rabbit myocytes, free PUFA reduced the amplitude of the calcium transient and attenuated the increase in calcium transient amplitude produced by noradrenaline. In addition, in the presence of PUFA noradrenaline did not prolong the AP and failed to induce EADs and DADs. Similar effects were detected during experiments on human cardiac myocytes derived from severe congestive cardiac failure hearts explanted during cardiac transplantation. The mechanisms responsible for the attenuation in adrenergic response are not fully understood. Szentandrássy et al. (2007) demonstrated that PUFA reduce cAMP levels but directly stimulate PKA. It is conceivable that during adrenergic stimulation PUFA significantly attenuate the increase in cAMP levels and therefore attenuate the response to adrenergic stimulation. In summary, these studies have consistently demonstrated that free PUFA *in vitro* exert profound effects on calcium handling at baseline and after adrenergic stimulation. These effects involve several components of the calcium handling system and produce marked reduction in calcium transient amplitude. These studies also suggest that free PUFA prevent DADs *in vitro* both by raising SR threshold for calcium waves (through inhibition of RyR) and by reducing the SR calcium content both before and after adrenergic stimulation. In addition, they inhibit sodium current and therefore prevent TA. It is unclear whether these effects also occur *in vivo* (see later).

EFFECTS OF MEMBRANE INCORPORATED PUFA

As mentioned above, dietary supplementation with EPA and DHA leads to their incorporation in the cardiac membrane phospholipids. Several studies have investigated the effects of incorporated PUFA but have reached conflicting reports. To simplify the subject we illustrate separately the effects of incorporated PUFA on calcium handling at baseline and following adrenergic stimulation.

EFFECTS ON CALCIUM HANDLING AT BASELINE

In 2001 Leifert et al. (2001) gave a DHA and EPA enriched diet to adult rats for 3 weeks. This diet increased the levels of incorporation of DHA (from 6 to 20% of total phospholipids) and EPA (from 0 to 3.2%). The incorporated PUFA did not affect calcium transient amplitude but decreased its rate of decay. In addition, incorporation of PUFA did not affect SR Ca content. The authors suggested that the reduction in calcium transient rate of decay was due to reduced NCX-mediated removal in the PUFA group. It is likely that the effects are more complex because isolated reduction of NCX function would have also increased SR calcium content. The Coronel group studied the effects of

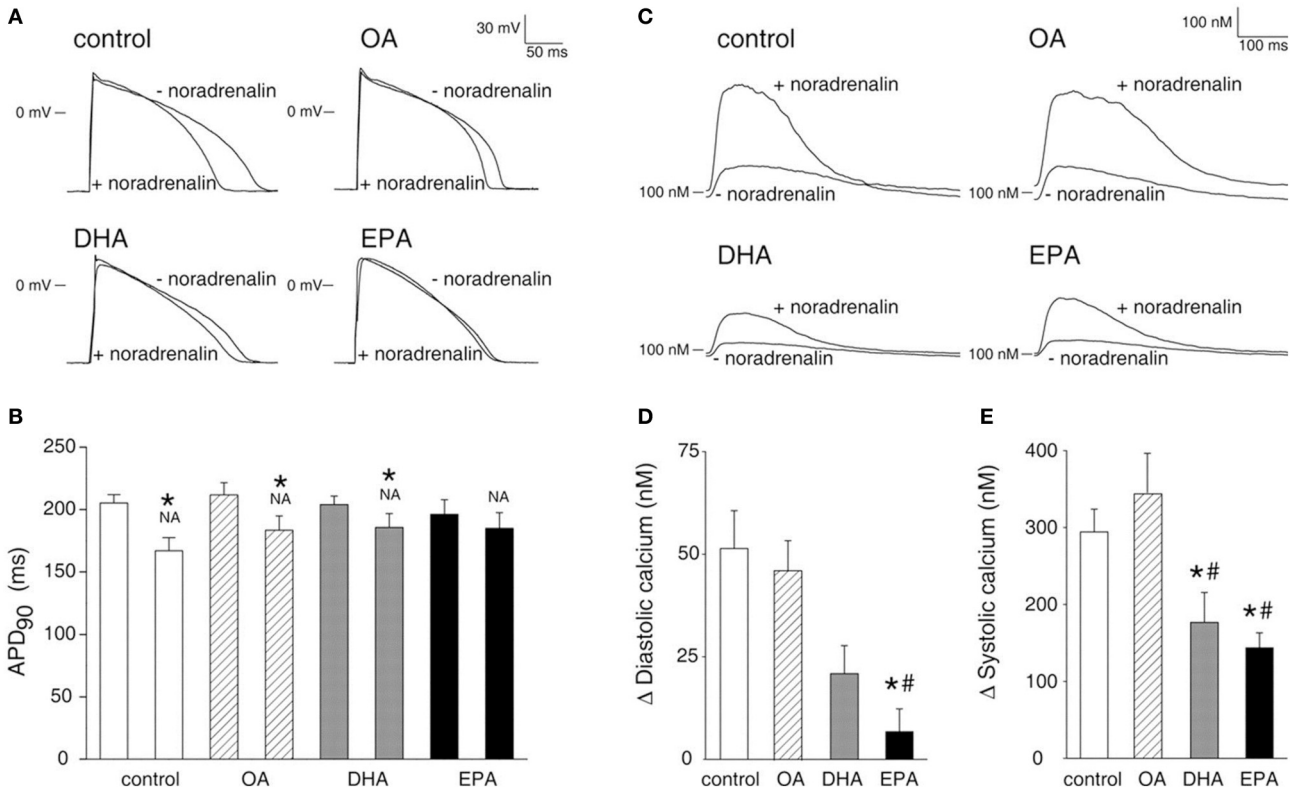

FIGURE 2 | PUFA reduce the sensitivity to noradrenalin. (A) Effects of free Oleic acid (OA), free EPA and free DHA on AP duration before and after application of noradrenalin. **(B)** Mean data for AP duration. $*P < 0.05$ indicates statistical differences with or without noradrenalin. **(C)** Effects of free OA free EPA and free DHA on calcium transient before and after application of noradrenalin. **(D)** Mean data for diastolic calcium levels. $*P < 0.05$ indicates statistical differences between DHA or EPA and control. **(E)** Mean data for peak systolic calcium levels. $\#P < 0.05$ indicates statistical differences between DHA or EPA and OA. Picture reproduced with permission from Den Ruijter et al. Circulation (2008) 117, 536–544.

a PUFA-enriched diet on pig ventricular myocytes. The diets were given for 8 weeks and produced a significant increase in the levels of incorporated DHA (from not detectable to around 7%) and EPA (from not detectable to around 15%). The most striking effect of incorporated PUFA was reduction in AP duration which was more prominent at slow frequencies and was mainly due to increase in two repolarizing currents: the slow component of the delayed rectifier current and inward rectifier K current (Den Ruijter et al., 2006; Verkerk et al., 2006). The analysis of calcium handling showed that the incorporated PUFA did not produce a significant effect on calcium transient amplitude but accelerated its rate of decay. SR calcium content was not affected but both L-type calcium current amplitude and NCX function were significantly reduced. The acceleration of the calcium transient rate of decay is probably explained by the shorter AP duration, but what is surprising is the fact that despite reduction in L-type calcium current amplitude, the calcium transient amplitude itself was not affected. Recently Billman et al. (2010) assessed the effects of DHA and EPA supplements (Omacor 1–2 or 4 tablets a day) on contractility and calcium transient in mixed breed dogs. The tablets were given for 3 months. The supplements produced a significant increase in EPA and DHA incorporation levels in cardiac membranes. However, the increased incorporation levels did not produce any significant effect on cardiac contractility, calcium transient amplitude and on L-type calcium current amplitude.

EFFECTS ON RESPONSE TO ADRENERGIC STIMULATION

Leifert et al. (2001) reported that incorporation of DHA and EPA in membranes of rat ventricular myocytes reduced the incidence of calcium waves and DADs during challenge with the β agonist isoproterenol. From the data illustrated by the authors, it is difficult to understand the mechanisms responsible for these protective effects. The authors also showed that the incorporation of DHA and EPA does not affect the increase in calcium transient amplitude produced by isoproterenol. Similarly to what described at baseline there was a reduction in the rate of decay of the calcium transient that was significant only at 0.2 Hz. From these data is difficult to establish how incorporated PUFA reduced the incidence of calcium waves. It is not clear whether PUFA affected SR threshold for calcium waves and/or SR calcium content. Similar to what was previously described by Leifert et al. in rat cardiac myocytes, the Coronel group demonstrated that incorporation of DHA and EPA in the membrane of pig ventricular myocytes reduced the incidence of calcium waves and DADs during challenge with norepinephrine (Berecki et al., 2007). They investigated in detail the mechanism responsible for these effects and demonstrated that membrane incorporation of PUFA

reduces the response to norepinephrine. In particular, incorporated PUFA blunted the increase in SR calcium content produced by norepinephrine and therefore attenuated the increase in calcium transient produced by norepinephrine. Incorporated PUFA also prevented the prolongation of AP potential produced by norepinephrine and it is unclear whether the blunting in the increase of SR calcium content is simply due to the effects on AP duration or also involves blunting of the stimulation of SERCA activity. In a recent paper, Billman et al. showed that incorporation of DHA and EPA in cardiac membranes of dogs did not affect the response to isoproterenol. The authors however did not investigate whether incorporated PUFA prevented the onset of calcium waves and DADs. However the finding that they do not attenuate response to isoproterenol would suggest that in these experiments they would not prevent DADs. An important issue that was not investigated by all these studies is whether incorporated PUFA have any effect on RyR and affect the threshold for SCR. Some studies inferred that because calcium transient amplitude was not affected there was no effect on RyR.

In summary, the studies that have investigated the effects of incorporated PUFA on Ca handling have reached conflicting conclusions. The cause of these differences remains unclear. Billman et al. have suggested that they may be related to the different species utilized in the studies. More specifically pigs (utilized by the Coronel Group) have a calcium-dependent transient outward current that is absent in dog and human myocytes. This could cause different effects of incorporated PUFA on AP duration and therefore SR calcium content before and after adrenergic stimulation. However this does not explain the differences in the effects of incorporated PUFA on the L-type Ca current. In addition, the pig study that documented attenuated adrenergic response used norepinephrine (β1 and α1 agonist) to produce adrenergic stimulation while the other two studies that documented limited effects utilized isoproterenol (β1and β2 agonist). This raises the possibility that stimulation with norepinephrine is more susceptible to modulation by incorporated PUFA. This is a possibility that needs to be investigated.

COMBINED EFFECTS OF FREE AND MEMBRANE INCORPORATED PUFA

One issue that has not been addressed by the studies performed is whether incorporation of PUFA in cardiac membrane affects the response to free PUFA. The issue is particularly important because diet supplementation leads both to membrane incorporation and to an increase in free circulating PUFA. Only one study has tried to address this issue (Den Ruijter et al., 2010) and has demonstrated that incorporation of PUFA in the membrane of rabbit myocytes shortens AP but prevents any further shortening when free PUFA are applied. Unfortunately this study has not determined whether the effects of free PUFA on calcium handling (inhibition of RyR and L-type calcium current) are affected by incorporated PUFA.enl.

DO THE EFFECTS OF PUFA ON CALCIUM HANDLING DETECTED *In vitro* OCCUR *In vivo* AS WELL?

The large amount of experimental evidence gained with *in vitro* experiments has not been supported by *in vivo* experiments. Several studies have demonstrated that *in vivo* both infusion of PUFA (free PUFA) (Billman et al., 1999) and chronic dietary supplementation (that leads to membrane incorporation and increased circulating free PUFA) protect from ischaemia-reperfusion related arrhythmias (McLennan et al., 1988; London et al., 2007). However a recent study evaluating the effects of dietary n-3 PUFA on susceptibility to post-myocardial infarction ventricular fibrillation in dogs, showed that despite significant increases in circulating as well as left ventricular PUFA levels, PUFA not only failed to prevent ischaemia-induced VF. Contrary to expectations dietary PUFA exerted pro-arrhythmic effects facilitating the onset of VF both in non-infarcted animals and in low-risk post-MI dogs that did not have VF prior to initiation of PUFA diet (Billman et al., 2012). These conflicting results highlight the fact that the pathogenesis of ischaemia reperfusion related arrhythmias is complex and certainly does not involve just alterations in calcium handling (Janse and Wit, 1989). Therefore this experimental evidence cannot be used as proof that PUFA modulate calcium handling *in vivo*. The evidence that PUFA *in vivo* modulate myocardial calcium handling is limited. This question could be answered by studies on cardiac contractility and on calcium handling related arrhythmias. To date no study has assessed the effects of acute PUFA administration on cardiac function and the two studies that have assessed the effects of PUFA diet supplementation on cardiac function have not confirmed the *in vitro* findings. In 1992, McLennan et al. (1992) demonstrated that dietary supplementation with PUFA in marmosets increased ejection fraction by enhancing LV filling. Recently Billman et al. (2010) studied the effects of PUFA supplementation on LV function (assessed by echo) and demonstrated that despite significant increases in the incorporation of DHA and EPA in the cardiac membrane (and probably in circulating free PUFA) there was no change in LV function. Interestingly these results are very different from what one would expect on the basis of *in vitro* studies that point more towards a reduction in calcium transient amplitude, cardiac contractility and LV function. These data suggest that *in vivo* there are complex modulating factors that mitigate or abolish the effects of PUFA observed *in vitro*. The evidence that PUFA prevent arrhythmias which are exclusively due to abnormalities in calcium handling, calcium waves and DADs (such as catecholaminergic and digoxin-related arrhythmias) is also limited. Only one study (Gudbjarnason et al., 1989) demonstrated that in rats a PUFA diet prevented the onset of VF following isoproterenol infusion. There is clearly a need for more targeted studies that specifically assess the anti-arrhythmic potential of PUFA in arrhythmia syndromes caused exclusively by abnormalities in calcium handling. To this purpose, a study in animal models and or patients with CPVT would address this point.

CONCLUSIONS

A large body of evidence gained from cellular experiments supports the idea that PUFA modulate myocardial calcium handling and exert anti-arrhythmic effect by preventing SCR and DADs. This large body of *in vitro* evidence still awaits confirmation by *in vivo* animal studies and clinical studies. Over the next decade targeted studies will tell us whether all these *in vitro* findings

also occur *in vivo* and whether PUFA are a treatment strategy for calcium handling related arrhythmias.

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Incorporated fish oil fatty acids prevent action potential shortening induced by circulating fish oil fatty acids

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Increased consumption of fatty fish, rich in omega-3-polyunsaturated fatty acids (ω3-PUFAs) reduces the severity and number of arrhythmias. Long-term ω3-PUFA-intake modulates the activity of several cardiac ion channels leading to cardiac action potential shortening. Circulating ω3-PUFAs in the bloodstream and incorporated ω3-PUFAs in the cardiac membrane have a different mechanism to shorten the action potential. It is, however, unknown whether circulating ω3-PUFAs in the bloodstream enhance or diminish the effects of incorporated ω3-PUFAs. In the present study, we address this issue. Rabbits were fed a diet rich in fish oil (ω3) or sunflower oil (ω9, as control) for 3 weeks. Ventricular myocytes were isolated by enzymatic dissociation and action potentials were measured using the perforated patch-clamp technique in the absence and presence of acutely administered ω3-PUFAs. Plasma of ω3 fed rabbits contained more free eicosapentaenoic acid (EPA) and isolated myocytes of ω3 fed rabbits contained higher amounts of both EPA and docosahexaenoic acid (DHA) in their sarcolemma compared to control. In the absence of acutely administered fatty acids, ω3 myocytes had a shorter action potential with a more negative plateau than ω9 myocytes. In the ω9 myocytes, but not in the ω3 myocytes, acute administration of a mixture of EPA + DHA shortened the action potential significantly. From these data we conclude that incorporated ω3-PUFAs into the sarcolemma and acutely administered ω3 fatty acids do not have a cumulative effect on action potential duration and morphology. As a consequence, patients with a high cardiac ω3-PUFA status will probably not benefit from short term ω3 supplementation as an antiarrhythmic therapy.

Keywords: fish oil, incorporated fish oil, diet, dietary fish oil, cardiac action potential

INTRODUCTION

The American Heart Association recommends to consume two portions of fish weekly, especially of fish rich in omega-3 poly-unsaturated fatty acids (ω3-PUFAs) (Kris-Etherton et al., 2002). This advice is based on a large body of evidence that shows that increased intake of fish oil fatty acids has favorable effects on cardiovascular disease outcomes (Burr et al., 1989; GISSI-Prevenzione Investigators, 1999; Yokoyama et al., 2007). A subanalysis of the GISSI Trial showed that an early and highly significant reduction of sudden cardiac death was the major component of total mortality reduction (Marchioli et al., 2002). Ventricular arrhythmias often precede sudden death and the effect of fish oil on cardiac arrhythmias has been extensively studied in humans and animals (Billman et al., 1999; Schrepf et al., 2004). Acute administration of ω3-PUFAs as well as long-term fish oil feeding experiments have been repeatedly shown to reduce the severity and number of arrhythmias (for review, Den Ruijter et al., 2007).

The antiarrhythmic effect of the main circulating ω3-PUFAs (eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA) is generally believed to be related to changes in cardiac cellular electrophysiology (Leaf et al., 2002). Configuration and duration of the cardiac action potential is highly relevant for arrhythmogenesis, regardless whether reentry or triggered activity constitutes the underlying mechanism (Den Ruijter et al. 2007). In general, both acutely administered and incorporated ω3 fatty acids shorten the action potential (Den Ruijter et al., 2006, 2007, 2008; Verkerk et al., 2006; Berecki et al., 2007). However, the ionic currents that are affected by incorporated ω3 fatty acids are different from those affected by acutely administered ω3 fatty acids. This suggests that an additive effect may result when both are present. In this study, we address the issue by measuring cardiac action potentials of isolated ventricular myocytes of rabbits fed a diet rich in fish oil (ω3) or sunflower oil (ω9) for 3 weeks. We superfused these myocytes with a mixture of ω3-PUFAs EPA and DHA. We conclude that acutely administered ω3-PUFAs cannot potentiate the effect of already incorporated ω3-PUFAs. Each leads to similar action potential changes.

MATERIALS AND METHODS

CELL PREPARATION

The investigation was approved by the local ethics committee and complied with the guiding principles of the Declaration of Helsinki. Male New Zealand White rabbits (4-months old) received a diet supplemented with either 2.5% fish oil or 2.5% high oleic sunflower oil as control, for 3 weeks (Verkerk et al., 2009).

After the feeding period, the animals were anesthetized by a combination of ketamine (intramuscular 100 mg) and xylazine (intramuscular 20 mg), heparinized (Heparine LEO 5000 IU),

and killed by an injection of pentobarbital (240 mg). The hearts were quickly excised and left ventricular midmyocardial cells were isolated by enzymatic dissociation as described previously (Den Ruijter et al., 2008). In short, hearts were mounted on a Langendorff perfusion apparatus and retrogradely perfused through the aorta with the following solutions: (1) Tyrode's solution for 15 min at a constant pressure (50 mm Hg), (2) a Ca^{2+}-free Tyrode's solution for 15 min (50 mm Hg), and (3) a Ca^{2+}-free Tyrode's solution to which collagenase type B (0.15 mg/ml, Boehringer Mannheim), collagenase type P (0.05 mg/ml, Boehringer Mannheim), trypsine inhibitor (0.1 mg/ml, Boehringer Mannheim), and 0.15 mg/ml hyaluronidase (Sigma, St. Louis, MO, USA) were added. During this last period, the ventricular free wall was perfused at a constant flow in a recirculating manner. When perfusion pressure dropped from an initial value of 50 to less than 2 mm Hg (usually after about 30 min), the left ventricular wall was cut into small pieces and further fractionated using a standard shaking protocol. All dissociation solutions were saturated with 100% O_2 and the temperature was maintained at 37°C.

Small aliquots of cell suspension were put in a recording chamber on the stage of an inverted microscope. Cells were allowed to adhere for 5 min after which superfusion with solution was started. This extracellular solution (36 ± 0.2°C) contained (in mmol/l): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.5, HEPES 5.0, pH 7.4 (NaOH). Quiescent rod-shaped myocytes with cross-striations and smooth surface were selected for measurements.

Throughout the manuscript N refers to the number of rabbits and n to the number of myocytes.

ELECTROPHYSIOLOGY

Action potentials were recorded with the amphotericin-perforated patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA). Data acquisition and analysis were accomplished using custom software. Signals were low-pass filtered with a cut-off frequency of 5 kHz and digitized at 10 kHz. Potentials were corrected for the estimated liquid junction potential. Patch pipettes (borosilicate glass; resistance ≈2.0 MΩ) contained (in mmol/l): K-gluconate 125, KCl 20, NaCl 5, amphotericin-B 0.22, HEPES 10, pH 7.2 (KOH).

Action potentials (APs) were elicited at 0.5–4 Hz by 3-ms, 1.5 times diastolic threshold current pulses through the patch pipette. We analyzed resting membrane potential (RMP), plateau potential (Pla) measured 100 ms after the AP upstroke, and AP duration (APD) at 20, 50 and 90% repolarization (APD_{20}, APD_{50}, and APD_{90}, respectively). Data from 10 consecutive APs were averaged.

Action Potentials were measured in the absence and presence of a clinically relevant mixture of fish oil fatty acids EPA (8 μmol/l) and DHA (7 μmol/l) (Den Ruijter et al., 2008) or in the absence and presence of the control fatty acid oleic acid (OA; 15 μmol/l). OA (Sigma), DHA (Sigma), and EPA (Sigma) were prepared as 10 mmol/l stock solutions in dimethyl sulfoxide, stored under nitrogen at –20°C, and diluted appropriately 20 min before use. In order to obtain steady-state conditions, AP recordings were started 5 min after application of the various fatty acids.

FATTY ACID ANALYSIS

Lipids were extracted from left ventricular tissue were extracted as described previously (Folch et al., 1957). Phospholipids were isolated with aminopropyl bonded phase columns (Bond Elut; Varian BV). Saponification and methylation of the phospholipids with boron trifluoride (Pierce, IL, USA) was performed and the formed fatty acid methyl esters were subjected to capillary gas chromatography using a Chrompack column (Fused Silica, Chrompack), a flame ionization detector, and H_2 as carrier gas. Fatty acid methyl esters were expressed as fraction of the total amount. Plasma free fatty acids were measured by gas-liquid chromatography (Püttmann et al., 1993).

STATISTICS

Data are presented as mean ± SEM. Group comparisons were made using the (un)paired *t*-test. ANOVA was used where pertinent. $P < 0.05$ defines statistical significance.

RESULTS

FISH OIL IN CARDIAC MEMBRANES AND PLASMA

The 3-week diet rich in fish oil resulted in a significant increase of ω3 fatty acids EPA and DHA of in the total amount of fatty acids extracted from the heart in the fish oil ω3 group (**Table 1**). The total amount of mono-unsaturated fatty acids, however, was significantly lower in the fish oil ω3 group compared to the sunflower oil ω9 group. Thus, ω3-PUFAs from the diet were incorporated in the cell membrane at the expense of mono-unsaturated fatty acids. In the plasma, free EPA levels were higher in the fish oil ω3 group compared to the sunflower oil ω9 group and the total amount of EPA + DHA was in the order of approximately 14 μmol/l. This amount is comparable to that measured in patients included in the study on omega-3 fatty acids and ventricular arrhythmia (SOFA) trial who were taking 2 g/day fish oil (Brouwer et al., 2006; Den Ruijter et al., 2008).

Table 1 | Phospholipid composition of the heart (% of total fat extracted) and plasma free fatty acid concentrations.

	ω3 diet (*N* = 5)	ω9 diet (*N* = 3)
PHOSPHOLIPIDS FROM VENTRICULAR HEART TISSUE (% OF TOTAL FAT EXTRACTED)		
Saturated fatty acids	29 (0.8)	29 (2.4)
Mono-unsaturated fatty acids	22 (2.0)*	31 (3.4)
Polyunsaturated fatty acids	47 (2.7)	38 (4.9)
Sum of ω3 fatty acids	14 (1.8)*	5 (0.1)
EPA	3.4 (0.9)*	0.1 (0.0)
DHA	4.7 (1.3)*	0.3 (0.2)
Sum of ω6 fatty acids	33 (1.1)	33 (5.1)
Unknown	1 (0.2)	1 (0.5)
PLASMA FREE FATTY ACIDS (μMOL/L)		
EPA	7.8 (1.01)*	3.1 (0.73)
DHA	5.9 (1.31)	4.6 (0.52)

*EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. *P < 0.05 compared to ω9 sunflower oil.*

INCORPORATED FISH OIL SHORTENS THE CARDIAC ACTION POTENTIAL

Figure 1A shows representative action potentials at 1 Hz from a myocyte isolated from a ω3 and a ω9 heart in the absence of fatty acids. The ω3 action potential has a more negative plateau potential (Pla) and is considerably shorter than the ω9 action potential. **Figure 1B** summarizes the action potential characteristics of the ω3 and ω9 myocytes. On average, ω3 myocytes show a 60% more negative plateau potential and a 20% shorter action potential at 90% repolarization. No significant differences in RMP were observed. The AP shortening in ω3 myocytes is evident at 0.5–3 Hz (**Figure 1C**).

ACUTELY ADMINISTERED FISH OIL FATTY ACIDS DO NOT SHORTEN THE CARDIAC ACTION POTENTIAL IN ISOLATED MYOCYTES OF FISH OIL FED RABBITS

To determine the effect of circulating ω3 fatty acids, we superfused myocytes of both groups with either a mixture of free EPA and DHA (combined 15 μM), or used the control fatty acid OA (15 μM). The concentration of fish oil fatty acids was based on the free fatty acid analysis of the plasma of the rabbits (**Table 1**).

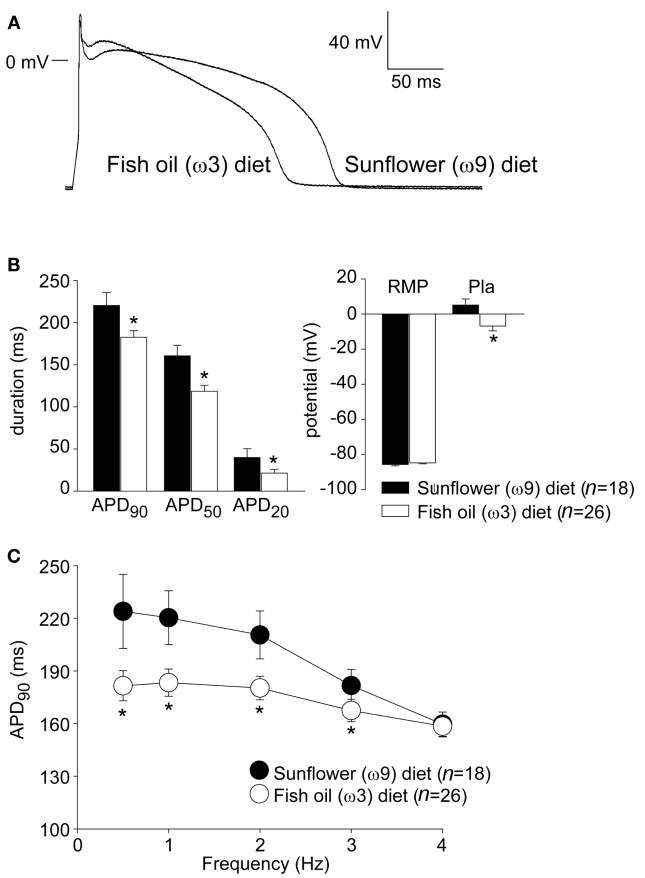

FIGURE 1 | (A) Typical examples of action potentials recorded at 1 Hz from isolated myocytes of a rabbit fed a ω3 diet and an ω9 diet. **(B)** Average action potential characteristics of ω3 and ω9 myocytes at 1 Hz. APD_{20}, APD_{50}, and APD_{90}, AP duration at 20, 50, and 90% repolarization; RMP, resting membrane potential; Pla, Plateau amplitude measured 100 ms following upstroke, $^*P < 0.05$ in unpaired *t*-test. **(C)** Stimulus frequency-dependency of APD_{90} in ω3 and ω9 myocytes.

Figures 2A,D show typical action potentials at 1 Hz recorded from an ω9 and an ω3 myocyte, respectively, in the absence and presence of the mixture of EPA + DHA. In the ω9 myocyte (**Figure 2A**), but not in the ω3 myocytes (**Figure 2D**), EPA + DHA shortened the action potential. **Figures 2B,E** summarize the effects of EPA + DHA on the APD_{90} in ω9 and ω3 myocytes, respectively. On average, ω9 myocytes have a 20% decreased APD_{90} in presence of EPA + DHA. In ω9 myocytes, the action potential shortening due to application of EPA + DHA was present at low pacing frequencies (**Figure 2C**). The control fatty acid OA did neither affect the action potential duration in ω9, nor in ω9 myocytes (**Figures 2C,F**).

DISCUSSION

The action potentials measured in isolated myocytes of the ω3 fed rabbits were approximately 20% shorter compared to the ω9 fed rabbits. Interestingly, the application of a mixture of physiological relevant concentrations of EPA + DHA resulted in a similar shortening of the action potential by approximately 20% in ω9 myocytes. The action potentials recorded from the ω3 fed rabbits were not shortened by acute application of EPA + DHA. These data indicate that it does not matter whether ω3-PUFAs are incorporated in the sarcolemma following a previous diet or are administered acutely with respect to their effects on action potentials. This is surprising because the effects of incorporated or acutely administered ω3-PUFAs on ionic currents are different, despite the similarity of the effects on action potential duration. For example, acute application of ω3-PUFAs in ferret cardiomyocytes does not change I_{K1} (Xiao et al., 2002), whereas incorporation of ω3-PUFAs following a fish oil diet increases I_{K1} by approximately 50% in porcine ventricular myocytes (Verkerk et al., 2006). Thus, although the mechanism may be different, both acute and incorporated ω3-PUFAs shorten the cardiac action potential duration of many species (Kang, 1995; Macleod et al., 1998; Ander et al., 2004).

The lack of shortening of EPA + DHA in the ω3 group implies that saturation of the membrane with ω3-PUFAs in the ω3 myocytes prevents further shortening of the cardiac action potential. This suggests that the mechanism for the cardiac action potential shortening in the ω9 group is not the result of a direct ligand-like interaction with the ion channels *per se*, but rather that it depends on membrane composition. However, Xiao et al. (2001) showed that substitution of a single amino acid in the hH1α unit of the fast sodium current (I_{Na}) reduced the inhibitory effect of EPA on the current amplitude, suggesting direct interference between the fatty acid and the ion channel. However, other ligand gated ion channels that lack amino acid homology with voltage gated ion channel are also inhibited by acute administration of ω3-PUFAs (Leaf et al., 2002). Therefore, it has been suggested that fatty acids primarily alter membrane composition close to ion channels rather than that they directly interact with the ion channel protein (Lundbaek and Andersen, 1999). The incorporation of the long acyl chain of the fatty acid may compress the phospholipid bilayer resulting in a mismatch with the hydrophobic length of the transmembrane channel (Girshman et al., 1997). Compression or stretch by the long-chain fatty acids may alter the conformational state and conductance of ion channels (Lundbaek and Andersen, 1999).

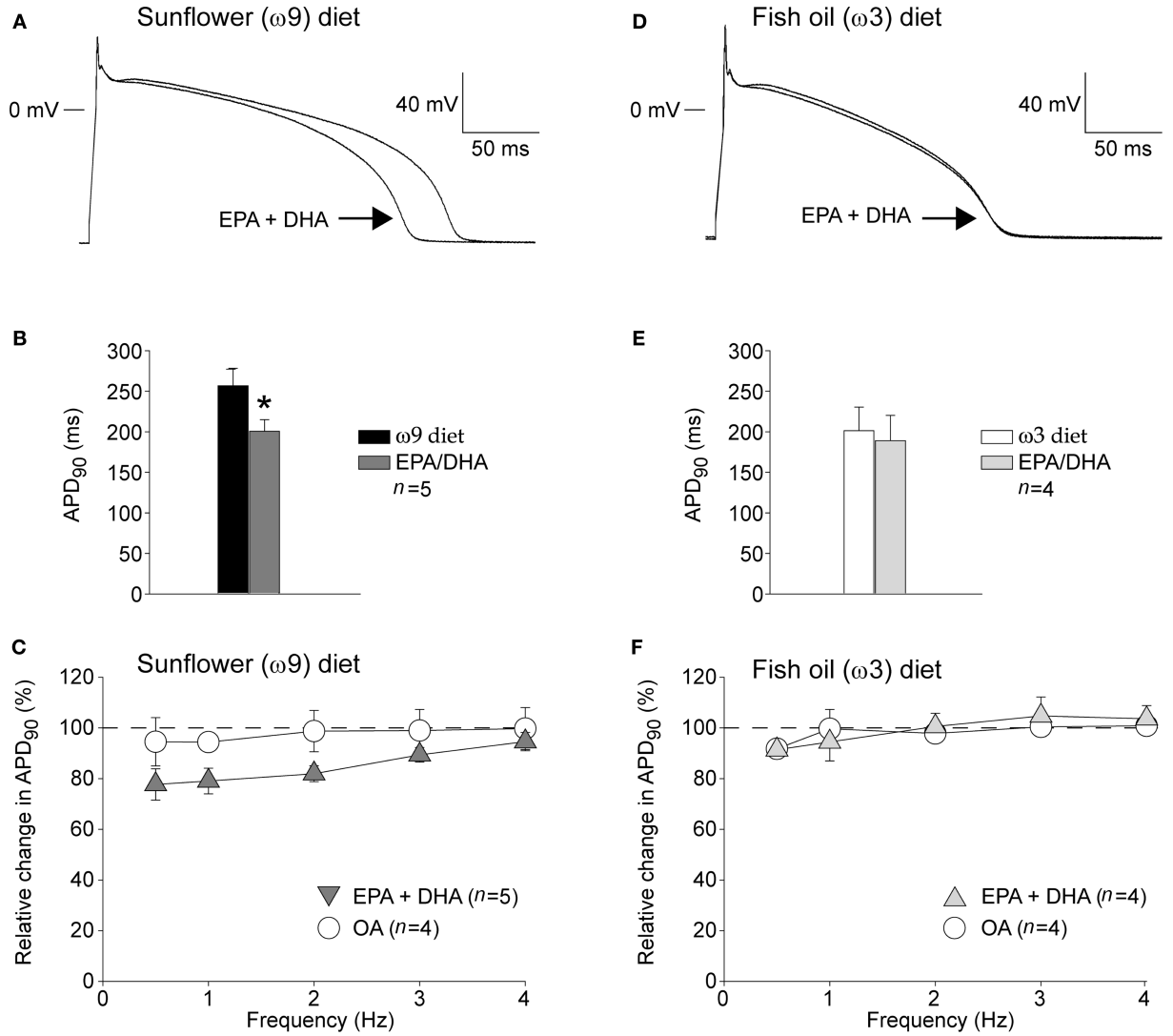

FIGURE 2 | (A,D) Typical example of a action potential recorded from a isolated myocytes of a rabbit fed a ω9 (A) or ω3 (D) diet in the absence and presence of EPA + DHA (15 μM in total). **(C,D)** Averaged data on APD_{90} in the ω9 **(B)** and ω3 **(E)** group before and after 5 min of superfusion with EPA + DHA. Superfusion with EPA/DHA resulted in a significant shortening of the APD_{90} in the ω9, but not in the ω3 group. Average data on the relative change induced by the acute superfusion of EPA + DHA and control fatty acid OA in the ω9 **(C)** and ω3 **(F)** group.

The effect of acute free ω3-PUFAs in our study occurs within minutes, an observation that suggests a rapid uptake into the outer leaflet of the membrane in a protein-independent manner. Long-term exposure to ω3-PUFAs may involve membrane fatty acid transporters resulting in esterification into phospholipids (see for review Glatz et al., 2010) and diffusion to specific domains. Here, ω3-PUFAs may indeed alter many basic properties of cardiac membranes (Stillwell and Wassall, 2003). Apparently, the acute and long-term exposures of ω3-PUFAs to the cardiac membrane, likely through different processes, influence each other.

Data on plasma levels of ω3-PUFAs following dietary interventions are limited. Fish oil supplements (2 g EPA and 1.4 g DHA) for 5 weeks in menopausal woman resulted in an increase in plasma EPA and DHA up to 0.5–0.7 mmol/l (Higdon et al., 2000). To which extent these fatty acids are "free" to enter the interstitial space is unknown. Therefore, we measured free fatty acid levels of EPA and DHA in plasma samples of the SOFA trial (Brouwer et al., 2006; Den Ruijter et al., 2008). This trial included patients who were taking fish oil-2g/day for a median of 365 days. Their free ω3-PUFAs were in the range of 5.0–16.4 μmol/l. The rabbits in our study had free EPA and DHA levels comparable to those seen in fish oil supplemented patients (**Table 1**).

Harris et al. (2004) have reported EPA and DHA levels in human cardiac tissue up to 2.5% of total fatty acids (Harris et al., 2004). Billman et al. (2010) reported a strong dose-dependent effect of fish oil intake (from 1 to 4 g/day) on cardiac omega-3 index (from 4 to 7%) in dogs. Also in humans, the cardiac omega-3 status can be modified. Moreover, several research groups advocate the use of the omega-3 red blood cells (the omega-3 index) as a measure of a person's cardiac omega-3 status

(Harris et al., 2004; von Schacky and Harris, 2007). Although we did not measure effects *in vivo*, our data suggest that patients with a high cardiac omega-3 status should maintain their status by continuation of their life style. They may not benefit from a high(er) dose of fish oil supplementation as a kind of prophylactic antiarrhythmic therapy.

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Docosahexaenoic acid reduces the incidence of early afterdepolarizations caused by oxidative stress in rabbit ventricular myocytes

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Accumulating evidence has suggested that ω3-polyunsaturated fatty acids (ω3-PUFAs) may have beneficial effects in the prevention/treatment of cardiovascular diseases, while controversies still remain regarding their anti-arrhythmic potential. It is not clear yet whether ω-3-PUFAs can suppress early afterdepolarizations (EADs) induced by oxidative stress. In the present study, we recorded action potentials using the patch-clamp technique in ventricular myocytes isolated from rabbit hearts. The treatment of myocytes with H_2O_2 (200 μM) prolonged AP durations and induced EADs, which were significantly suppressed by docosahexaenoic acid (DHA, 10 or 25 μM; $n = 8$). To reveal the ionic mechanisms, we examined the effects of DHA on L-type calcium currents ($I_{Ca.L}$), late sodium (I_{Na}), and transient outward potassium currents (I_{to}) in ventricular myocytes pretreated with H_2O_2. H_2O_2 (200 μM) increased $I_{Ca.L}$ by 46.4% from control (-8.4 ± 1.4 pA/pF) to a peak level (-12.3 ± 1.8 pA/pF, $n = 6$, $p < 0.01$) after 6 min of H_2O_2 perfusion. H_2O_2-enhanced $I_{Ca.L}$ was significantly reduced by DHA (25 μM; -7.1 ± 0.9 pA/pF, $n = 6$, $p < 0.01$). Similarly, H_2O_2-increased the late I_{Na} (-3.2 ± 0.3 pC) from control level (-0.7 ± 0.1 pC). DHA (25 μM) completely reversed the H_2O_2-induced increase in late I_{Na} (to -0.8 ± 0.2 pC, $n = 5$). H_2O_2 also increased the peak amplitude of and the steady state I_{to} from 8.9 ± 1.0 and 2.16 ± 0.25 pA/pF to 12.8 ± 1.21 and 3.13 ± 0.47 pA/pF respectively ($n = 6$, $p < 0.01$, however, treatment with DHA (25 μM) did not produce significant effects on current amplitudes and dynamics of I_{to} altered by H_2O_2. In addition, DHA (25 μM) did not affect the increase of intracellular reactive oxygen species (ROS) levels induced by H_2O_2 in rabbit ventricular myocytes. These findings demonstrate that DHA suppresses exogenous H_2O_2-induced EADs mainly by modulating membrane ion channel functions, while its direct effect on ROS may play a less prominent role.

Keywords: docosahexaenoic acid, H_2O_2, early afterdepolarizations, reactive oxygen species, L-type calcium channel, sodium channel

INTRODUCTION

Extensive studies on the potential effects of fish oil omega-3 poly unsaturated fatty acids (ω-3 PUFA) on cardiac rhythm have provided controversial results (von Schacky, 2008). While some interventional studies reported either no effect or even promotion of arrhythmias in some subgroups of patients with heart disease (Raitt et al., 2005; Coronel et al., 2007; Den Ruijter et al., 2007; Cheng and Santoni, 2008), other studies have reported beneficial effects of ω-3-PUFAs on cardiac rhythm resulting in a reduction in the incidence of sudden cardiac death or mortality (London et al., 2007; Cheng and Santoni, 2008; Nodari et al., 2009). It seems that fish oil fatty acids may exert either pro- or anti-arrhythmic effects, probably depending on different underlying mechanisms for the arrhythmias. Recent studies have also shown ω-3-PUFAs suppress afterdepolarizations and triggered activities induced by K channel blockers or by β-adrenergic stimulation in failing hearts (Den Ruijter et al., 2006, 2008; Berecki et al., 2007; Smith et al., 2009). However, it is unclear whether ω-3-PUFAs have protective effects on arrhythmias induced by oxidative stress. Reactive oxygen species (ROS) have recently been implicated in the pathogenesis of cardiac arrhythmia during ischemic-reperfusion, aging, and heart failure. Oxidative stress caused by exogenous H_2O_2 induces early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) that may in turn trigger lethal arrhythmias. These afterdepolarizations are a result of a net increase in inward current, which is induced by activation of late sodium current (I_{Na}) and the L-type calcium current (I_{CaL}) via oxidized Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII; Ward and Giles, 1997; Xie et al., 2009; Zhao et al., 2011). Our most recent study suggested that the transient outward potassium current (I_{to}) may also facilitate EAD generation by H_2O_2 (Zhao et al., 2012b). In the present study, we aim to assess the effects of docosahexaenoic acid (DHA, one of

ω-3-PUFAs) on exogenous H_2O_2-induced EADs, and to further reveal potential underlying ionic mechanisms.

MATERIALS AND METHODS

This investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996). All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey-New Jersey Medical School and by the Ethical Committee of Xi'an Jiaotong University. All experiments were performed at 35–37°C.

CELL ISOLATION

Ventricular myocytes were enzymatically isolated from the hearts of New Zealand white rabbits (male, 1.8–2.5 kg) as described previously (Xie et al., 2009). Briefly, after rabbits were anesthetized with intravenous pentobarbital hearts were removed and perfused retrogradely at 37°C in Langendorff fashion with nominally Ca^{2+}-free Tyrode's solution containing 1.4 mg/mL collagenase (Type II; Worthington) and 0.1 mg/ml protease (type XIV, Sigma) for 25–30 min. The hearts were removed from the perfusion apparatus after washing out the enzyme solution, the left ventricles were gently teased apart with forceps in a Petri dish and the myocytes were filtered through a nylon mesh. The Ca^{2+} concentration was gradually increased to 1.8 mM, and the cells were stored at room temperature and used within 8 h.

ELECTROPHYSIOLOGICAL RECORDING

Myocytes were current-or voltage-clamped using the perforated whole-cell patch-clamp technique (240 μg/ml amphotericin B; Rae et al., 1991) for recordings of action potential, or $I_{Ca.L}$, I_{to}, and late I_{Na}. Voltage or current signals were measured with a MultiClamp 700A patch-clamp amplifier controlled by a personal computer using a Digidata 1322 acquisition board driven by pCLAMP 10 software (Molecular Devices, Sunnyvale, CA, USA).

To record action potentials (APs), patch pipettes (resistance 2–4 MΩ) were filled with an internal solution containing (in mM): 110 K-aspartate, 30 KCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 Na_2-phosphocreatine, 0.05 cAMP, pH was adjusted to 7.2 with KOH. The cells were superfused with Tyrode's solution containing (in mM): 136 NaCl, 4.0 KCl, 0.33 Na_2PO_4, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH was adjusted to 7.4 with NaOH. APs were elicited with 2 ms, 2 to 4 nA square pulses at a pacing cycle length (PCL) of 6 s.

To record the $I_{Ca,L}$, patch pipettes (2–4 MΩ) were filled with an internal solution containing (in mM): 110 Cs-Aspartate, 30 CsCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 Na_2-phosphocreatine, 0.05 cAMP, pH 7.2 with CsOH, and the cells were perfused with a modified Tyrode's solution, in which KCl was replaced with CsCl. The myocytes were stimulated at a PCL of 6 s with a double-pulse protocol. Following a 100-ms prepulse to −40 mV from the holding potential of −80 mV (to inactivate Na^+ current and T-type Ca^{2+} current), $I_{Ca.L}$ was elicited by a subsequent test depolarization step to 0 mV for 300 ms.

Late I_{Na} was measured as described previously (Song et al., 2006). Glass pipettes (1–2 MΩ) were filled with an internal solution containing (in mM): 110 Cs-Aspartate, 30 CsCl, 10 HEPES, 0.5 EGTA, 0.2 Na_3-GTP, 5 Na_2-phosphocreatine-, 5 MgATP, pH 7.2 was adjusted with CsOH. Myocytes were bathed with a modified Tyrode's solution in which KCl was replaced with CsCl. Nifedipine (30 μM) was added to the bath solution to block calcium channels. Late I_{Na} was elicited by 300 ms voltage-clamp pulses from −90 to −30 mV at a PCL of 6 s from a holding potential of −80 mV.

To record I_{to}, the pipette and superfusion solutions were the same as those for AP recording. Tetrodotoxin (TTX, 10 μM) and $CdCl_2$ (0.5 mM) were added into the Tyrode's solution to inhibit I_{Na} and $I_{Ca,L}$. I_{to} was evoked by 400 ms depolarizing pulses to test potentials between −40 and +50 mV (0.1 Hz). The holding potential was set at −80 mV and a 100 ms prepulse was applied to −60 mV to inactivate the I_{Na}. I_{to} recovery from inactivation was investigated using a conventional two-pulse protocol: an inactivating pulse depolarizing to +50 mV for 400 ms (P1) followed by a variable recovery interval and subsequent +50 mV test pulse (P2). The inactivation of I_{to} and recovery from inactivation were best fit with a double exponential equation. All electrophysiological data were normalized as current densities by dividing measured current amplitude by whole-cell capacitance.

All chemicals were purchased from Sigma-Aldrich unless indicated. Because DHA is very sensitive to oxidation, DHA (Sigma-Aldrich) was dissolved in 100% ethanol under N_2 and kept at −20°C in the dark. Immediately before use, the DHA stock solution was diluted in the bath solution to reach the final concentrations needed. The maximum final concentration (0.1%) of ethanol had no effect on membrane currents.

MEASUREMENT OF INTRACELLULAR ROS

The myocytes were incubated with 5 μM C-DCDHF-DA-AM (Invitrogen) for 30 min. C-DCDHF- DA is oxidized by ROS to dichlorofluorescein (DCF). ROS fluorescence (emission: ∼530 nm) was measured by a 200 ms-exposure (excitation: ∼480 nm) every 30 s using the Andor Ixon charge-coupled device camera. Recordings were started after a stable baseline was achieved.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Differences were tested for statistical significance by using paired or unpaired Student's t tests, with $p < 0.05$ considered significant.

RESULTS

DHA SUPPRESSES THE EADs INDUCED BY H_2O_2

Action potentials were recorded from single ventricular myocytes isolated from rabbit hearts using the perforated whole-cell patch-clamp technique under current-clamp mode. In order to reliably induce EADs, the cells were paced at a PCL of 6 s based on our previous studies (Sato et al., 2009; Xie et al., 2009; Zhao et al., 2012a). The average APD_{90} of rabbit ventricular myocytes is 266 ± 23 ms ($n = 8$) at base line. After APD and morphology reached steady state, the cells were perfused with 200 μM H_2O_2 until EADs consistently appeared. Consecutively, DHA at either 10 or 25 μM was added in the presence of H_2O_2. The sudden and dramatic increase in APD_{90} in **Figure 1A** indicates the incidence of EADs. As shown in **Figures 1A,B**, EADs were consistently induced by H_2O_2 at 5 min after perfusion. DHA (25 μM) shortened the APD

prolongation from 894 ± 78 ms to 278 ± 52 ms, and significantly suppressed the frequency of EADs induced by H_2O_2. The incidence of EADs was assessed by counting the number of EADs within 10 APs (from eight cells) in control, after H_2O_2 (200 μM) and H_2O_2 (200 μM) + DHA (at 10 or 25 μM). The incidence of EAD was suppressed in all tested cells ($n = 8$), five of which showed complete abolishment of EADs after 3–5 min of treatment with 25 μM DHA. As summarized in **Figure 1C**, the incidence of H_2O_2-induced EADs were significantly reduced by direct perfusion of DHA at both 10 and 25 μM, in a dose-dependent manner ($p < 0.05$ and $p < 0.01$, respectively, Fisher's exact test).

INHIBITORY EFFECT OF DHA ON I_{CAL} ENHANCED BY H_2O_2

Our previous studies have shown that reactivation of $I_{Ca.L}$ plays a key role in H_2O_2-induced EAD in rabbit ventricular myocytes (Xie et al., 2009; Song et al., 2010). Therefore, we first assessed the potential involvement of $I_{Ca.L}$ in the inhibitory effect of DHA on H_2O_2-induced EADs. $I_{Ca.L}$ was recorded in rabbit ventricular myocytes using the perforated whole-cell patch-clamp technique under voltage-clamp mode. As shown in **Figure 2A**, H_2O_2 (200 μM) gradually increased the amplitude of $I_{Ca.L}$ at both peak and late phases (at ∼ 250 ms), which reached the steady state at 5–7 min, consistent with the time course for EAD induction as shown in **Figure 1**. The I-V relations for the peak current (**Figure 2B**) showed that the $I_{Ca.L}$ amplitude was pronouncedly increased at testing potentials −10 to +40 mV. For example a 46.4% enhancement was caused at 0 mV, i.e., from −8.4 ± 1.4 to −12.3 ± 1.8 pA/pF ($n = 6$, $p < 0.01$). DHA (25 μM) significantly suppressed/reversed the elevation of the $I_{Ca,L}$ amplitude (e.g., to −7.1 ± 0.9 pA/pF at 0 mV; $n = 6$, $p < 0.01$ compared to H_2O_2-induced effect). In order to test the DHA effect on $I_{Ca.L}$ under normal membrane potential conditions, we also performed AP-clamp experiments. As shown in **Figures 2C,D**, DHA markedly decreased both the peak and the late phase of $I_{Ca.L,}$ which were enhanced by H_2O_2, under AP-clamp conditions.

INHIBITORY EFFECT OF DHA ON LATE SODIUM CURRENT INCREASED BY H_2O_2

Since the activation of late I_{Na} also contributes to EAD generation induced by H_2O_2 (Ward and Giles, 1997; Xie et al., 2009), we next evaluated the effect of DHA on H_2O_2-enhanced late $I_{Na.}$ Late I_{Na} was elicited by 300 ms voltage-clamp pulses from −90 to −30 mV at a PCL of 6 s. The magnitude of late I_{Na} was evaluated by integration of the area (nA × ms = pC) of the current over the last 50 ms of the −30 mV depolarizing pulse, using the integration (area) feature of the pCLAMP program. As shown in **Figure 3**, the late current component was significantly enhanced by H_2O_2 (200 μM) from −0.7 ± 0.1 pC to −3.2 ± 0.3 pC ($n = 5$, $p < 0.01$) at 4–6 min after perfusion, when it reaches steady state level. This elevation was completely suppressed by Tetrodotoxin (TTX, 10 μM), a selective I_{Na} inhibitor, confirming this late sustained inward current is due to late I_{Na}, although we cannot exclude minor contaminations on the baseline current from other currents such as Na-Ca exchange current (I_{NCX}), $I_{Ca,L}$ or leaky

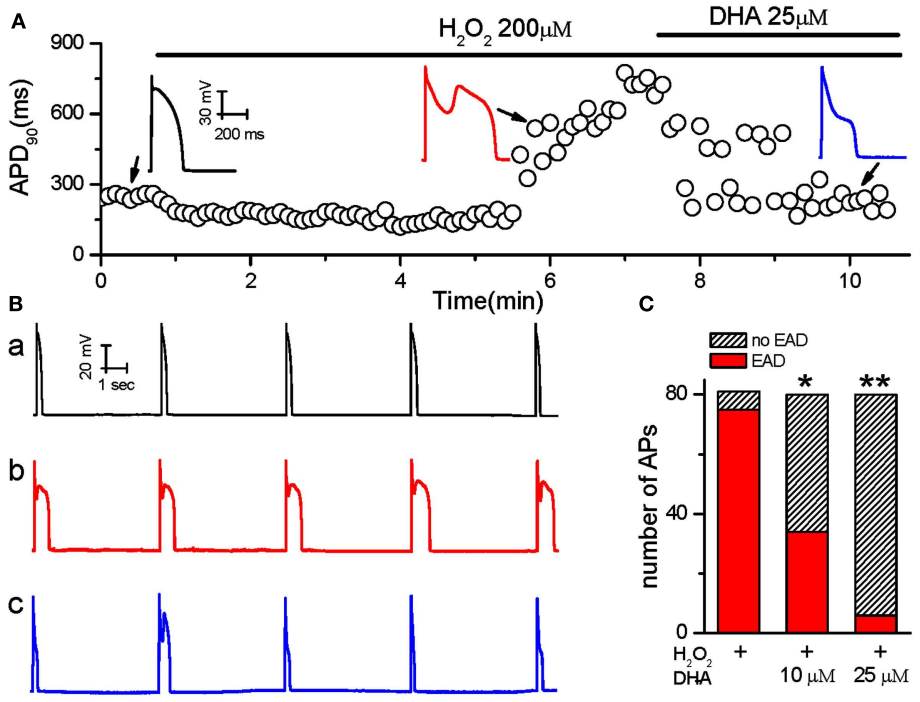

FIGURE 1 | The inhibitory effects of DHA on Early afterdepolarizations (EADs) induced by H_2O_2. (A) Values of consecutive APD_{90} are plotted over time. The ventricular myocyte was treated with H_2O_2 and DHA as indicated by the horizontal bars above the plot. Three representative AP recordings under different conditions are shown in the insets. **(B)** Five consecutive AP recordings from a cell exposed to control perfusate **(a)**, 200 μM H_2O_2 **(b)** and 200 μM H_2O_2 + 25 μM DHA **(c)**. **(C)** Summarized bar graph showing dose-dependent inhibitory effects of DHA on the incidence of EADs induced by H_2O_2 ($n = 8$ cells). $^*p < 0.05$, $^{**}p < 0.01$; Fisher's Exact Test vs. H_2O_2.

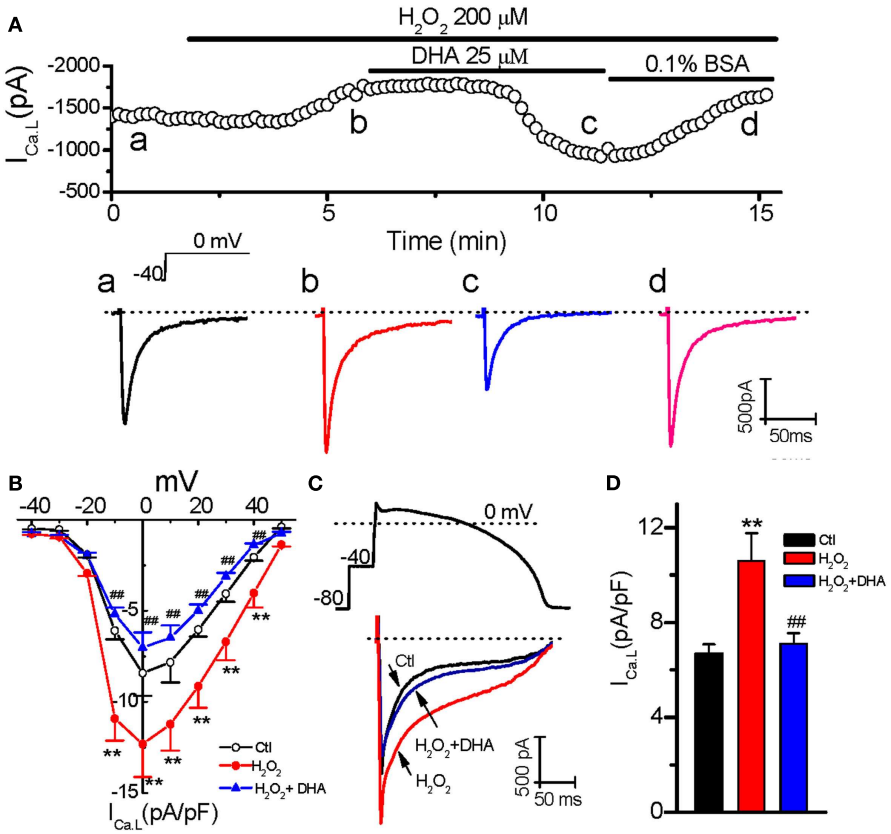

FIGURE 2 | Inhibitory effects of DHA on $I_{Ca.L}$ enhanced by H_2O_2. (A) Time course of peak $I_{Ca,L}$ in a myocyte treated with 200 μM H_2O_2 in the absence and presence of 25 μM DHA, and 0.1% bovine serum albumin (BSA). Representative traces of $I_{Ca,L}$ corresponding to points a–d are shown under the plot. **(B)** The current-voltage relations for peak $I_{Ca,L}$ from six cells treated with 200 μM H_2O_2 in the absence and presence of 25 μM DHA. Test potentials ranged from −40 to +50 mV in 10 mV steps. **(C)** An AP-clamp waveform (above) and superimposed current traces showing $I_{Ca,L}$ under control (Ctl), in the presence of 200 μM H_2O_2, and DHA(25 μM) + H_2O_2 are shown respectively. **(D)** The late phase currents measured at 30-150 ms after the upstroke in **(C)** were summarized showing an inhibitory effect of DHA on the enhancement of $I_{Ca,L}$ by H_2O_2 ($n = 6$). $^{**}p < 0.01$ vs. control; ## $p < 0.01$ vs. H_2O_2 group.

current. H_2O_2-increased late I_{Na} was effectively attenuated by 25 μM DHA (to -0.8 ± 0.2 pC at 2–4 min after DHA application, $n = 5$, $p < 0.01$).

EFFECT OF DHA ON I_{To} INCREASED BY H_2O_2

Consistent with our recent finding (Zhao et al., 2012b), H_2O_2 (200 μM) increased the amplitudes of both peak (from 8.94 ± 1.07 to 12.8 ± 1.21 pA/pF at testing potential of 50 mV, $n = 6$, $p < 0.01$) and steady state (late phase at the end of 400 ms pulse; from 2.16 ± 0.25 to 3.13 ± 0.47 pA/pF, $n = 6$, $p < 0.01$) component of I_{to}. Additionally, H_2O_2 also slowed inactivation ($\tau_{s,in}$ from 96.6 ± 4.3 to 158.1 ± 5.7 ms; $\tau_{f,in}$ from 17.4 ± 1.7 to 24.7 ± 14.0 ms, $n = 7$, $p < 0.01$). However, DHA at 25 μM, the concentration which dramatically suppressed H_2O_2-induced EADs, did not show any significant effects on current amplitudes (peak $I_{to} = 12.51 \pm 1.47$ pA/pF; $I_{to.ss} = 3.34 \pm 0.31$ pA/pF, $n = 6$, $p > 0.05$ compared to H_2O_2, respectively) or inactivation process of I_{to} ($\tau_{s,in}$: 154.6 ± 6.6 ms and $\tau_{f,in}$: 23.9 ± 1.1 ms, $n = 7$, $p > 0.05$ compared to H_2O_2; **Figures 4A–C**). Furthermore, we found that H_2O_2 accelerated the recovery from inactivation of I_{to} mainly by decreasing the fast component ($\tau_{f,re}$: from 817.2 ± 79.2 ms to 341.9 ± 26.1 ms, $n = 7$, $p < 0.05$), but not by changing the slow component ($\tau_{s.re}$: from control 5335.4 ± 504.8 ms to H_2O_2 4963.2 ± 459.9 ms, $p > 0.05$). Similarly DHA (25 μM) did not cause any significant alteration in I_{to} recovery kinetics after H_2O_2 treatment (**Figure 4D**).

EFFECT OF DHA ON INTRACELLULAR ROS LEVELS

The level of oxidative stress may either increase or decrease in tissues from humans and animals supplemented with fish oil as reported previously (Garrido et al., 1989; Mas et al., 2010; Tsuduki et al., 2011). To determine whether DHA reduces the incidence of EAD via affecting (decreasing) intracellular ROS, the effect of DHA on intracellular ROS levels was measured in isolated ventricular myocytes treated with exogenous H_2O_2 (200 μM) by monitoring CM-DCF fluorescence intensity. The effect of DHA on intracellular ROS levels in the absence of H_2O_2 was also measured. As shown in **Figure 5**, exogenous H_2O_2 produced a rapid and dramatic increase in DCF fluorescence intensity in the myocytes and the F/F_0 of DCF fluorescence intensity reached a steady state value of 2.18 ± 0.24 at 6–10 min after H_2O_2 treatment. However, DHA (25 μM, either pretreatment or after treatment) showed no

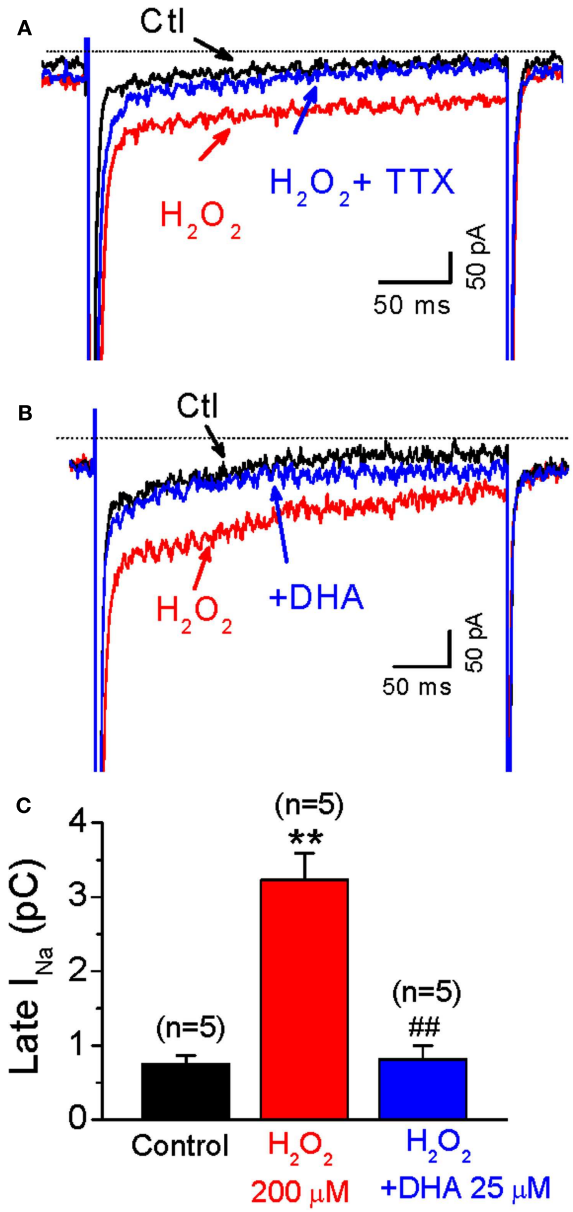

FIGURE 3 | Inhibitory effects of DHA on late I_{Na} enhanced by H_2O_2. **(A)** Representative I_{Na} traces under control condition (Ctl), in the presence of 200 μM H_2O, and H_2O_2 + TTX (10 μM), respectively. **(B)** Representative I_{Na} traces under control condition (Ctl), in the presence of 200 μM H_2O, and H_2O_2 + DHA (25 μM), respectively. **(C)** A bar graph summarizing 200 μM H_2O_2-induced increase of late I_{Na}, which is significantly suppressed by 25 μM DHA. $^{**}p < 0.01$ vs. control; ## $p < 0.01$ vs. H_2O_2 group.

significant effect on the fluorescence of CM-DCF either in the absence or presence of H_2O_2 (2.21 ± 0.33, $n = 8$, $p > 0.05$).

DISCUSSION

Experimental and clinical studies have obtained controversial results regarding the effects of fish oil or ω-3 PUFA on cardiac rhythm (von Schacky, 2008). Differences in the underlying pathogenic mechanisms for the arrhythmia in differing patient groups or animal models may account for these controversies. We and others have previously shown that both exogenous and endogenous ROS-induced EADs can serve as triggers for arrhythmias. In the present study, we provide the first evidence showing that DHA attenuates EADs induced by H_2O_2.

The molecular and ionic mechanisms of ion channel modulation by DHA are still not completely understood. A recent review article comprehensively summarized the potential antiarrhythmogenic electrophysiological effect of ω3-PUFAs on the heart (Richardson et al., 2011). Inhibitory effects of DHA on EADs may involve multifactorial mechanisms e.g., (1) via ROS modulation. Although ω3-PUFA may slightly increase levels of oxidative stress due to the susceptibility to oxidation, low to moderate ROS exposure can conversely give rise to up-regulation of antioxidant enzymes and increase antioxidant ability (scavenging ROS) in cardiac tissue (Jahangiri et al., 2006); (2) via direct modulation of ion channels by binding to the channels or affecting cell membrane lipid properties (such as membrane lipid peroxidation). While there is a widespread effect of ω3-PUFA on ion channels and ion pumps, Ca^{2+} and Na^{+} currents are most sensitive to ω3-PUFAs (Richardson et al., 2011). Nevertheless, our present data suggest that the ionic mechanisms underlying inhibitory effect of DHA on EADs most likely involve the direct inhibition on the $I_{Ca,L}$ and late I_{Na} rather than its putative antioxidant ability. This notion was supported by the observation that there was no effect on CM-DCF fluorescence induced by DHA at the same concentration that led to reduction of EADs. In addition, the fast time course for DHA suppression of $I_{Ca,L}$ and late I_{Na} also supports a mechanism of direct inhibition of ion channels by DHA. Our most recent data showed H_2O_2 also activates I_{to} and may facilitate EAD generation (Zhao et al., 2012b). In the present study, however, we showed that DHA did not reverse the I_{to} activated by H_2O_2 in rabbit ventricular myocytes, which is inconsistent with previous reports that DHA markedly reduces I_{to} in human atrial cells and rat ventricular myocytes even at lower concentrations (5–10 μM; Bogdanov et al., 1998; Verkerk et al., 2006; Li et al., 2009). We do not have a ready explanation for this discrepancy, while the molecular subtypes of I_{to} proteins might be different between rabbits and other species (including humans) or between different locations in the heart (e.g., ventricle vs. atria). In addition, the H_2O_2-activated I_{to} seemed to be more resistant to DHA than the I_{to} at baseline, since we observed the inhibitory effects of 25 μM DHA on I_{to} (up to ∼50%) in the absence of H_2O_2.

It has also been reported that n–3-PUFAs are capable of reducing the activity of CaMKII (Zaloga et al., 2006), which may partially account for the inhibitory effect of DHA on EADs. However, since DHA does not alter the ROS level in the presence of H_2O_2 (**Figure 5**), the reduction of CaMKII activity, if any, may be mediated by less Ca entry secondarily to $I_{Ca,L}$ blockage, rather than by lower oxidation. Further experiments are needed identify the involvement of CaMKII.

Nevertheless, our present study suggests fish oil supplements may be effective in preventing/treating arrhythmias under an increased oxidative stress condition and serve as an alternative or complimentary anti-arrhythmic drug. Conditions with elevated oxidative stress level including ischemia/reperfusion, heart failure and aging might benefit from fish oil supplements.

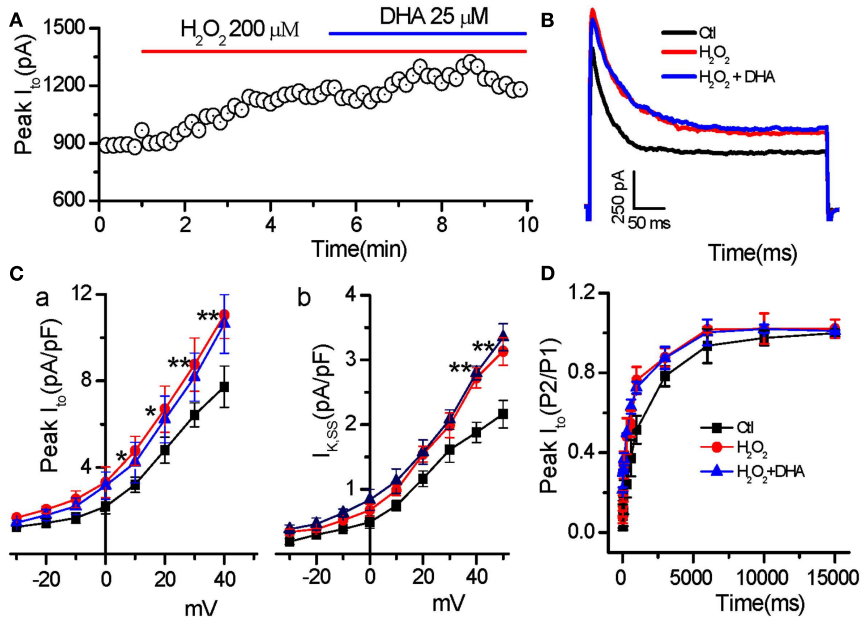

FIGURE 4 | Less effect of DHA on I_{to} enhanced by H_2O_2. (A) Time course of peak I_{to} in a myocyte treated with H_2O_2 in the absence and presence of DHA. **(B)** Representative traces of the I_{to} under control, in the presence of H_2O_2 (200 μM), and H_2O_2 + DHA (25 μM), respectively. **(C)** Current–voltage relations of the peak I_{to} **(C-a)** and steady state currents ($I_{K,SS}$, **C-b**) showing less effects of DHA on enhancement of peak I_{to} and $I_{K,ss}$ ($n = 6$, $^*p < 0.05$, $^{**}p < 0.01$ vs. control.). Test potentials ranged from −60 to +50 mV in 10 mV steps. **(D)** Recovery of I_{to} from inactivation showing no significant effect of DHA (25 μM) on the I_{to} recovery sped-up by H_2O_2 (200 μM; $p > 0.05$, $n = 7$).

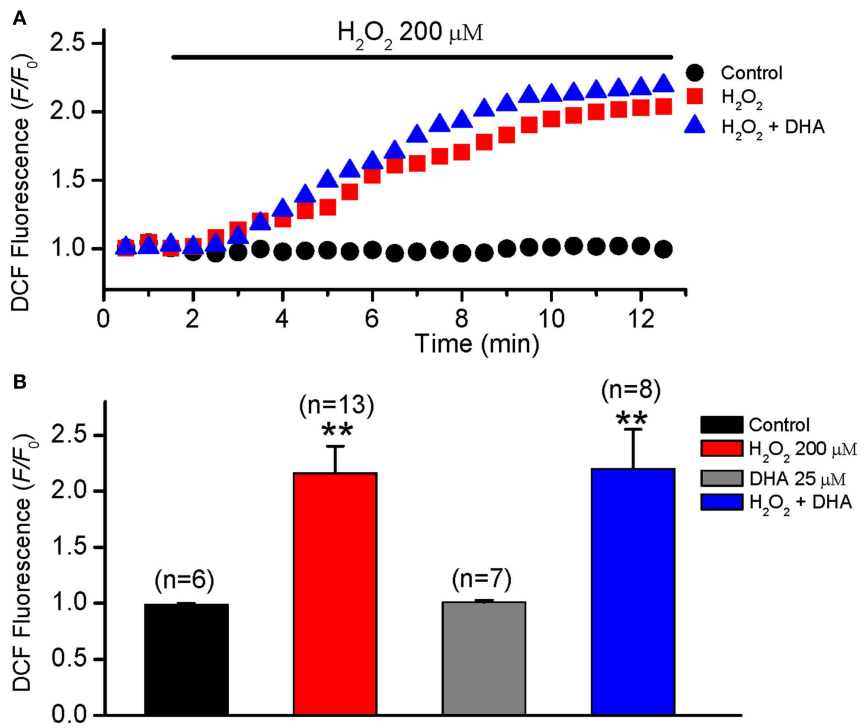

FIGURE 5 | No effect of DHA on ROS levels in isolated rabbit ventricular myocytes. ROS levels were measured by monitoring DCF fluorescence intensity in isolated myocytes every 30 s in control, H_2O_2 (200 μM) and H_2O_2 + DHA (25 μM) groups. **(A)** Time courses of DCF fluorescence intensity (F/F_0) in three representative myocytes from the three groups, respectively. **(B)** Histograms summarizing the DCF intensities for each group measured at 6 min after treatment of H_2O_2. $^{**}p < 0.01$ compared to control. Numbers in parentheses indicate the number of cells in each group.

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Dietary omega-3 polyunsaturated fatty acids suppress NHE-1 upregulation in a rabbit model of volume- and pressure-overload

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Background: Increased consumption of omega-3 polyunsaturated fatty acids (ω3-PUFAs) from fish oil (FO) may have cardioprotective effects during ischemia/reperfusion, hypertrophy, and heart failure (HF). The cardiac Na^+/H^+-exchanger (NHE-1) is a key mediator for these detrimental cardiac conditions. Consequently, chronic NHE-1 inhibition appears to be a promising pharmacological tool for prevention and treatment. Acute application of the FO ω3-PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibit the NHE-1 in isolated cardiomyocytes. We studied the effects of a diet enriched with ω3-PUFAs on the NHE-1 activity in healthy rabbits and in a rabbit model of HF induced by volume- and pressure-overload. **Methods:** Rabbits were allocated to four groups. The first two groups consisted of healthy rabbits, which were fed either a diet containing 1.25% (w/w) FO (ω3-PUFAs), or 1.25% high-oleic sunflower oil (ω9-MUFAs) as control. The second two groups were also allocated to either a diet containing ω3-PUFAs or ω9-MUFAs, but underwent volume- and pressure-overload to induce HF. Ventricular myocytes were isolated by enzymatic dissociation and used for intracellular pH (pH_i) and patch-clamp measurements. NHE-1 activity was measured in HEPES-buffered conditions as recovery rate from acidosis due to ammonium prepulses. **Results:** In healthy rabbits, NHE-1 activity in ω9-MUFAs and ω3-PUFAs myocytes was not significantly different. Volume- and pressure-overload in rabbits increased the NHE-1 activity in ω9-MUFAs myocytes, but not in ω3-PUFAs myocytes, resulting in a significantly lower NHE-1 activity in myocytes of ω3-PUFA fed HF rabbits. The susceptibility to induced delayed afterdepolarizations (DADs), a cellular mechanism of arrhythmias, was lower in myocytes of HF animals fed ω3-PUFAs compared to myocytes of HF animals fed ω9-MUFAs. In our rabbit HF model, the degree of hypertrophy was similar in the ω3-PUFAs group compared to the ω9-MUFAs group. **Conclusion:** Dietary ω3-PUFAs from FO suppress upregulation of the NHE-1 activity and lower the incidence of DADs in our rabbit model of volume- and pressure-overload.

Keywords: Na^+/H^+-exchanger, pH_i, fish oil, diet, heart failure, hypertrophy, arrhythmias

INTRODUCTION

Increased consumption of omega-3 polyunsaturated fatty acids (ω3-PUFAs) from fish oil (FO) may exert beneficial effects on the heart, as evidenced by a decreased risk of ischemic heart disease, sudden cardiac death (Burr et al., 1989; GISSI-Prevenzione Investigators, 1999), and a lower incidence of heart failure (HF; Mozaffarian et al., 2005; Yamagishi et al., 2008; Levitan et al., 2009; Chen et al., 2011). Various mechanisms for the observed beneficial effects of ω3-PUFAs have been proposed, i.e., decrease in blood pressure, heart rate, and platelet aggregation, anti-inflammatory (Kris-Etherton et al., 2002), and ionic remodeling resulting in a decrease of cardiac arrhythmias (den Ruijter et al., 2007; London et al., 2007), but the exact mechanisms are not fully known.

Evidence is increasing that the Na^+/H^+-exchanger isoform-1 (NHE-1) plays a crucial role in ischemia/reperfusion injury, hypertrophy, and HF (for reviews, see Cingolani and Ennis, 2007; Fliegel, 2009; Vaughan-Jones et al., 2009). The NHE-1 is an integral membrane protein that extrudes one H^+ ion in exchange for one Na^+ ion in an electroneutral fashion. Its activity is high at acidic intracellular pH (pH_i) conditions and gradually declines to zero when its set-point pH_i value, just above resting pH_i value (~pH 7.2) is reached. At resting pH_i acid extrusion through NHE-1 activity equals acid loading activity and proton production rate, thereby maintaining pH_i at neutral values. This, however, is at the expense of a continuous Na^+ influx. Thus, the NHE-1 has also a major role in intracellular Na^+ ($[Na^+]_i$) loading (Baartscheer and van Borren, 2008; Fliegel, 2009; Vaughan-Jones et al., 2009).

This $[Na^+]_i$ loading effect is of importance especially under conditions where NHE-1 activity is high such as ischemia/reperfusion (Ayoub et al., 2003; Bak and Ingwall, 2003; van Borren et al., 2004), hypertrophy, and HF (Baartscheer et al., 2003a; Chahine et al., 2005; van Borren et al., 2006; Nakamura et al., 2008). In these conditions, the $[Na^+]_i$ loading via the NHE-1 shifts the driving force of Na^+/Ca^{2+} exchange into the direction of less forward and increased reversed modes, which consequently will elevate intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration with potentially detrimental cardiac effects. Consequently, a reduction of Na^+ influx via NHE-1 inhibition appears to be a promising pharmacological tool for the treatment of ischemia/reperfusion, hypertrophy, and HF (Baartscheer et al., 2005; Cingolani and Ennis, 2007).

Goel et al. (2002) have shown that acute application of the ω3-PUFA eicosapentaenoic acid (EPA) as well as docosahexaenoic acid (DHA) inhibited the NHE-1 in isolated cardiomyocytes. Considering the importance of NHE-1 in ischemia/reperfusion injury, hypertrophy, and HF, NHE-1 inhibition may be the crucial link between FO and the well-known cardioprotective effects of ω3-PUFAs. In addition, it suggests that ω3-PUFAs may be an alternative or a complementary approach to existing NHE-1 inhibiting pharmacological drugs. In the present study we assessed the effects of long term treatment with ω3-PUFAs on NHE-1 in healthy rabbits and in a rabbit model of volume- and pressure-overload. To specifically address the effects of a diet rich in ω3-PUFAs from FO on NHE-1 in our study, we chose to use the ω9-MUFAs as a control fatty acids. These fatty are more abundantly present in the human diet and do not alter cardiac electrophysiology (den Ruijter et al., 2008). Therefore, rabbits were fed a diet rich in either ω3-PUFAs from FO or omega-9 monounsaturated fatty acids (ω9-MUFAs) from high-oleic sunflower oil (HOSF) as control.

MATERIALS AND METHODS

ANIMALS AND DIET

All experiments were carried out in accordance with guidelines of the local institutional animal care and use committee. In addition, the investigation conforms the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Male New Zealand White rabbits (4 months old) received a diet (150 g/day; Research Diet Services, Wijk bij Duurstede, Netherlands) supplemented with either 1.25% (w/w) FO or 1.25% HOSF as control. Food consumption of every rabbit was measured and average food intake did not differ between FO and HOSF fed animals (data not shown). In the HF model, diet started 1 week before the surgical procedures to induce HF (see below). Lipids from the diet and the left ventricular tissue were extracted with the method of Folch et al. (1957). **Table 1** summarizes the fatty acid composition of these diets. In short, the total PUFA content was higher in the ω3-PUFAs diet due to a larger amount of both EPA and DHA.

Heart failure was induced by combined volume- and pressure-overload in two sequential surgical procedures as described previously in detail (Vermeulen et al., 1994; Baartscheer et al., 2003a; Verkerk et al., 2007). In short, volume overload was produced by catheter-induced damage to the aortic valve until pulse pressure was increased by about 100%. Three weeks later, pressure-overload was created by abdominal aortic stenosis by ligation of approximately 50%. After 3 weeks for the healthy animals and after 4 months for the HF animals, the rabbits were anesthetized [(ketamine (50 mg i.m.) and xylazine (10 mg i.m.)], heparinized (5000 IU), and killed by intravenous injection of pentobarbital (240 mg).

Table 1 | Fatty acid composition of ω9-MUFA and ω3-PUFA diets.

	ω9-MUFA	ω3-PUFA
SATURATED FATTY ACIDS		
Total	16.2	21.8
MONOUNSATURATED FATTY ACIDS		
Total	44.0	17.9
C18:1ω9 (oleic acid)	42.4	12.2
POLYUNSATURATED FATTY ACIDS		
Total	37.6	56.6
C18:2ω6 (LA)	30.5	26.8
C18:3ω3 (ALA)	6.85	7.12
C20:4ω6 (AA)	0.00	0.57
C20:5ω3 (EPA)	0.1	9.2
C22:6ω3 (DHA)	0.1	6.3
Other, unidentified fatty acids	2.2	3.6

Fatty acid composition expressed as percentage of total fatty acids. ω9-MUFA, high-oleic sunflower oil; ω3-PUFA, fish oil; LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. The sum of listed components is less than the totals indicated here, since not all components were analyzed.

CELL PREPARATION

Single midmyocardial myocytes were isolated by enzymatic dissociation from the most apical part of the left ventricular free wall as described previously (de Groot et al., 2003). Small aliquots of cell suspension were put in a recording chamber on the stage of an inverted microscope. Myocytes were allowed to adhere for 5 min after which superfusion with Tyrode's solution was started. Tyrode's solution (36 ± 0.2°C) contained (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.5, HEPES 5.0, pH 7.4 (NaOH). Quiescent rod-shaped cross-striated myocytes with a smooth surface were selected for measurements.

INTRACELLULAR pH MEASUREMENTS

Intracellular pH (pH_i) was measured in carboxy-seminaphthorhodafluor-1 (SNARF-AM, Molecular Probes) loaded myocytes as described previously (Baartscheer et al., 2003a; van Borren et al., 2004). In short, myocytes were excited at 515 nm (75 W Xenon arc lamp) and dual wavelength emission of SNARF was recorded at wavelengths of 580 nm (I_{580}) and 640 nm (I_{640}). A rectangular adjustable slit ensured negligible background fluorescence levels. As shown in a typical example in **Figure 1A**, the I_{580}/I_{640} ratio was calibrated by a series of precisely set pH solutions that contained 140 mM K^+ instead of Na^+ and the K^+/H^+ ionophore nigericin (10 μM; Sigma). **Figure 1B** shows the resulting calibration curve were the 580/640 ratios were plot against the pH_i.

Intrinsic buffering power

In general, activities of acid loaders or extruders are expressed as the amount of acid or base extruded or loaded per second, the

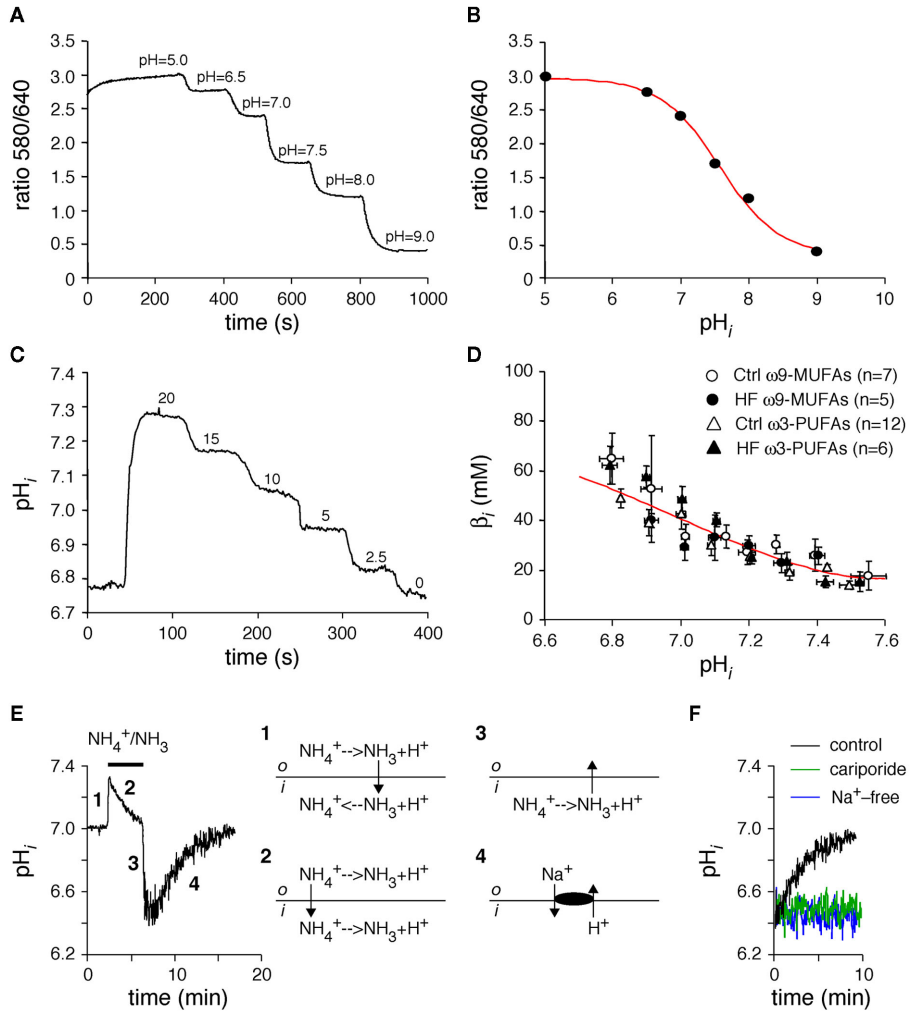

FIGURE 1 | (A,B) *In vivo* calibration curve of SNARF-AM. To determine calibration curve myocytes were loaded with SNARF-AM and superfused in presence op nigericin with several high K^+ solutions at various pH_o values **(A)**. When the external and internal K^+ free concentrations are equal, pH_i is the same as pH_o. The calibration curve **(B)** was obtained by plotting the ratios (580/640) against the corresponding pH_o. The red line represents a Henderson–Hasselbalch fit through these data, which revealed a maximum ratio of 2.97 and a minimum ratio of 0.36 and a pK_a of 7.57. **(C,D)** Determination of the intrinsic sarcoplasmic buffer power (β_i). Typical example of the "stepwise reduction in extracellular NH_3/NH_4^+ approach" in a myocyte isolated from a healthy, ω3-PUFA fed animal **(C)**, and pH_i–β^i relationships of ω3-PUFA and ω9-MUFA myocytes of both healthy rabbits (Ctrl) and the rabbits with heart failure (HF). **(E)** Typical example and schematic explanation of an ammonium prepulse. **(F)** Typical examples of effects of Na^+-free conditions and cariporide on the acid load recovery in HEPES-buffered conditions.

proton flux (J_H; Roos and Boron, 1981). Changes in pH_i are not linearly related to J_H due to the presence of a pH_i-dependent intrinsic sarcoplasmic buffer power (β_i). β_i was determined by the "stepwise reduction in extracellular NH_3/NH_4^+ approach" as described previously (Boyarsky et al., 1988), and shown in the typical example of **Figure 1C**. With each stepwise decrease in extracellular NH_3/NH_4^+, the amount of protons delivered to the cytoplasm (Δ[acid]i) was considered equal to the resultant change in intracellular NH_4^+ concentration, which can be calculated from the observed pH_i. ΔpH_i was taken as the change in pH_i produced by the stepwise decrease in extracellular NH_3/NH_4^+. β_i was then calculated as $-\Delta[acid]_i/\Delta pH_i$ (Roos and Boron, 1981). β_i was assigned to the mean of the two pH_i values used for its calculation. **Figure 1D** shows the pH_i–β_i relationships of myocytes isolated from ω3-PUFA and ω9-MUFA fed healthy rabbits and of myocytes of ω3-PUFA and ω9-MUFA fed rabbits with model of volume- and pressure-overload. The pH_i–β_i relationships did not differ significantly, indicating that neither the diets nor HF affect the β_i.

NHE-1 activity

Na^+/H^+-exchanger isoform-1 activity was measured in HEPES-buffered conditions as recovery rate from acidosis due to ammonium prepulses as described previously (van Borren et al., 2004). **Figure 1E** shows a typical example and explanation of the pH changes in response to an ammonium prepulse. In short, 20 mM NH_4Cl (NH_4^+/NH_3) was rapidly added to the Tyrode's solution resulting instantly in alkalinization of myocytes (**Figure 1E**, phase

1), after which they slowly recovered from alkalization mainly because of NH_4^+ influx (**Figure 1E**, phase 2). After withdrawal of NH_4^+/NH_3 from the extracellular solutions all intracellular NH_4^+ is converted to NH_3 which leaves the myocyte and the remaining H^+ acidifies the sarcoplasm (**Figure 1E**, phase 3). Subsequently, in HEPES-buffered solutions, the myocytes slowly recovered from the acid load due to NHE-1 activity (**Figure 1E**, phase 4). The recovery from acid load is Na^+-dependent as well as blocked by cariporide (10 μM; **Figure 1F**), typical hallmarks of the NHE-1. From the pH_i traces we computed the dpH_i/dt's and multiplied these with β_i to arrive at $J_{NHE\text{-}1}$.

CELLULAR ELECTROPHYSIOLOGY

Action potentials (APs) and delayed afterdepolarizations (DADs) were recorded with the perforated patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Signals were low-pass filtered with a cut-off frequency of 2 kHz and digitized at 3 kHz. Data acquisition and analysis were accomplished using custom software and potentials were corrected for liquid junction potential (Barry and Lynch, 1991). APs were elicited at 3 Hz by 3-ms long, 1.2× threshold current pulses through the patch pipette. We analyzed resting membrane potential (RMP), maximal upstroke velocity (V_{max}), AP amplitude (APA), and AP duration at 20, 50, and 90% repolarization (APD_{20}, APD_{50}, and APD_{90}, respectively). Susceptibility to DADs were evoked by a 3-Hz (10-s) rapid pacing episode followed by an 8-s pause (tracing period) in the presence of norepinephrine (100 nM, Centrafarm, Etten-Leur, The Netherlands). A DAD was defined as a temporary, short-lived deviation from (an otherwise stable) RMP of more than 2 mV. Data from five APs and five rapid pacing episode were averaged. Cell membrane capacitance, an electrophysiological measure of cell size, was estimated as we described previously in detail (Verkerk et al., 2004).

STATISTICS

Data are mean ± SEM. Groups were compared using Two-Way Repeated Measures ANOVA followed by pairwise comparison using the Student–Newman–Keuls test, Fisher's exact test, or unpaired *t*-test. $P < 0.05$ is considered statistical significant.

RESULTS

ω3-PUFA RICH DIET RESULTS IN ω3-PUFAs INCORPORATED IN THE CELL MEMBRANE

The diet rich in ω3-PUFAs from FO resulted in a significant increase of ω3-PUFAs EPA and DHA of the total amount of fatty acids extracted from the heart of both healthy and HF rabbits (**Table 2**). The total amount of monounsaturated fatty acids, however, was significantly lower in the ω3-PUFAs fed rabbit hearts compared to the ω9-MUFAs fed rabbit hearts. Thus, ω3-PUFAs from the diet were incorporated in the cell membrane at the expense of monounsaturated fatty acids.

DIETARY ω3-PUFAs DO NOT AFFECT NHE-1 ACTIVITY IN HEALTHY RABBITS

In a first series of experiments, we measured the NHE-1 in myocytes isolated from healthy rabbits. Body weight after 3 weeks of diet was similar in ω3-PUFA and ω9-MUFA fed animals (3.1 ± 0.2, $n = 9$) vs. 2.9 ± 0.2 kg ($n = 7$), $P > 0.05$). **Figure 2A**, top panel, shows representative recordings of the pH_i recovery after an ammonium prepulse (see Materials and Methods) in a myocyte isolated from an ω3-PUFA and ω9-MUFA fed rabbit. The pH_i recovery, and consequently the calculated $J_{NHE\text{-}1}$ (**Figure 2A**, bottom panel), was virtually overlapping in the myocytes of ω3-PUFA and ω9-MUFA fed animals. **Figure 2B** shows the average $J_{NHE\text{-}1}$ in the myocytes of ω3-PUFA ω9-MUFA fed animals. The average $J_{NHE\text{-}1}$ was not significantly different in the myocytes of ω3-PUFA and ω9-MUFA fed animals at any of the pH_i's.

Table 2 | Phospholipid composition of the heart (% of total fat extracted).

	Healthy rabbits		HF rabbits	
	ω9-MUFA ($n = 3$)	**ω3-PUFA ($n = 5$)**	**ω9-MUFA ($n = 10$)**	**ω3-PUFA ($n = 8$)**
SATURATED FATTY ACIDS				
Total	29 ± 3	29 ± 1	23 ± 1	25 ± 1
MONOUNSATURATED FATTY ACIDS				
Total	31 ± 4	22 ± 2*	23 ± 2	17 ± 1*
C18:1ω9 (oleic acid)	26 ± 4	17 ± 2*	19 ± 2	13 ± 1*
POLYUNSATURATED FATTY ACIDS				
Total	38 ± 6	47 ± 3	47 ± 1	51 ± 1*
C18:2ω6 (LA)	28 ± 3	28 ± 1	32 ± 1	30 ± 1
C18:3ω3 (ALA)	3.8 ± 0.8	4.0 ± 0.6	2.8 ± 0.5	3.2 ± 0.4
C20:4ω6 (AA)	4.7 ± 3.2	4.6 ± 1.5	8.9 ± 1.2	5.7 ± 0.6
C20:5ω3 (EPA)	0.1 ± 0.1	3.4 ± 1*	0.1 ± 0.1	3.9 ± 0.3*
C22:6ω3 (DHA)	0.3 ± 0.2	4.7 ± 1.4*	0.8 ± 0.2	6.4 ± 0.5*
Unidentified fatty acids	1.4 ± 0.6	1.4 ± 0.3	7.4 ± 1.1	7.2 ± 0.9

*Fatty acid composition expressed as percentage of total fatty acids. ω9-MUFA, high-oleic sunflower oil; ω3-PUFA, fish oil; LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. The sum of listed components is less than the totals indicated here, since not all components were analyzed. *P < 0.05 ω9-MUFA vs. 3-PUFA diet.*

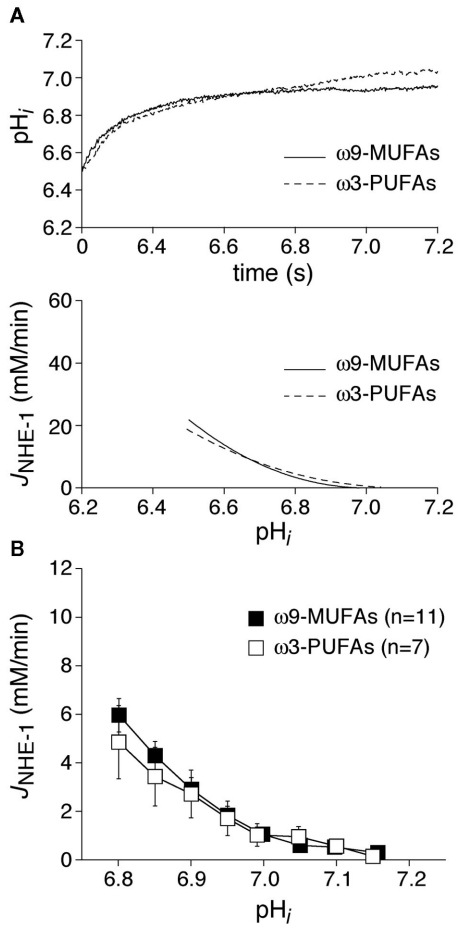

FIGURE 2 | (A) Typical examples of pH_i recovery after an ammonium prepulse (top) and the calculated J_{NHE-1} (bottom) in an ω9-MUFA and ω3-PUFA myocyte isolated from healthy animals. **(B)** Average J_{NHE-1} in myocytes of ω9-MUFA and ω3-PUFA fed healthy animals.

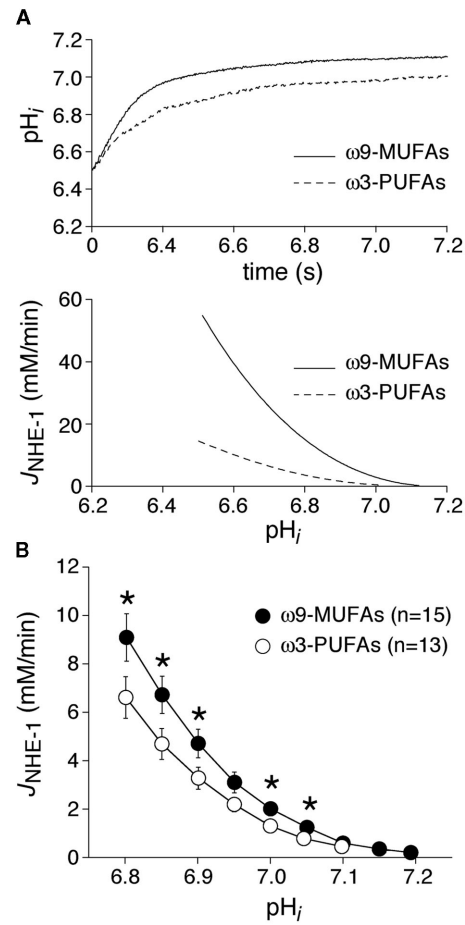

FIGURE 3 | (A) Typical examples of pH_i recovery after an ammonium prepulse (top) and the calculated J_{NHE-1} (bottom) in an ω9-MUFA and ω3-PUFA myocyte isolated from rabbit which underwent volume- and pressure-overload. **(B)** Average J_{NHE-1} in myocytes isolated from ω9-MUFA and ω3-PUFA fed rabbits with volume- and pressure-overload. $*P < 0.05$.

DIETARY ω3-PUFAs REDUCES THE NHE-1 ACTIVITY IN A RABBIT MODEL OF VOLUME- AND PRESSURE-OVERLOAD

In a second series of experiments, we studied the NHE-1 activity in myocytes isolated from rabbits that underwent model of volume- and pressure-overload for 4 months. **Figure 3A**, top panel, shows representative of the pH_i recovery after an ammonium prepulse in a myocyte of an ω3-PUFA and ω9-MUFA fed animal. In the HF rabbits, pH_i recovery after an ammonium prepulse was slower in the myocyte of the ω3-PUFA animal compared to that in the myocyte of the ω9-MUFA fed animal. Consequently, the calculated J_{NHE-1} was lower in the myocyte of the ω3-PUFA rabbit (**Figure 3A**, bottom panel). **Figure 3B** shows the average J_{NHE-1} in myocytes of ω3-PUFA and ω9-MUFA fed HF animals. The average J_{NHE-1} was significantly lower in myocytes of ω3-PUFA animals at pH values lower than 7.1 ($P < 0.05$).

DIETARY ω3-PUFAs OPPOSE THE INCREASE IN NHE-1 ACTIVITY INDUCED BY HEART FAILURE

In HF animals, but not in healthy animals, NHE-1 activity in ω3-PUFAs myocytes was significantly lower than in ω9-MUFAs myocytes (**Figures 2B** and **3B**). Previous studies demonstrate that the NHE-1 activity is significantly increased in animal and patients with HF (Baartscheer et al., 2003a; Chahine et al., 2005; van Borren et al., 2006). This suggests that dietary ω3-PUFAs suppress the increase in NHE-1 activity in our rabbit HF model. **Figure 4** shows the averages J_{NHE-1} at pH 7.0 of myocytes of ω3-PUFA and ω9-MUFA fed healthy and HF animals. HF significantly increased the J_{NHE-1} in myocytes of ω9-MUFA fed animals ($P < 0.05$), but not in myocytes of ω3-PUFA fed animals. Thus, a diet rich in ω3-PUFAs suppresses the increase in NHE-1 activity associated with HF.

DIETARY ω3-PUFAs REDUCES THE INCIDENCE OF DADs IN A RABBIT MODEL OF VOLUME- AND PRESSURE-OVERLOAD

Volume- and pressure-overload in rabbit increased the NHE-1 activity resulting in elevated $[Na^+]_i$ and secondarily to increased $[Ca^{2+}]_i$ (Baartscheer et al., 2003a). The altered Ca^{2+} handling in HF is associated with spontaneous Ca^{2+} release from the sarcoplasmic reticulum (SR; Baartscheer et al., 2003b), which activate

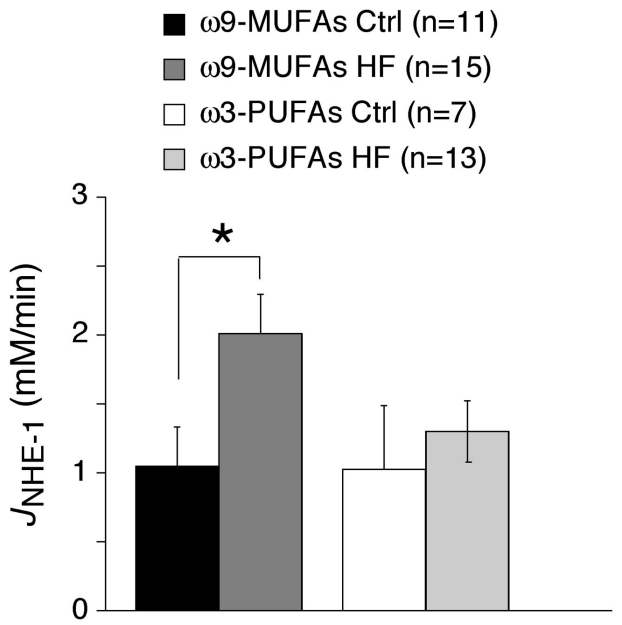

FIGURE 4 | Average J_{NHE-1} at pH = 7.0 of healthy (Ctrl) and HF rabbits fed ω9-MUFA and ω3-PUFA rich diets. Note that J_{NHE-1} is increased in the HF model in ω9-MUFA fed animals, while it was not changed in ω3-PUFA animals. $^*P < 0.05$.

the transient inward current, *I*ti, resulting in DADs (Verkerk et al., 2000a). ω3-PUFAs, and not ω9-MUFA, suppress the increase in NHE-1 activity in our rabbit model of volume- and pressure-overload. Thus, we hypothesized that the incidence of DADs is lower in myocytes of ω3-PUFAs fed HF rabbits compared to those of ω9-MUFA fed HF rabbits. Next, we tested the susceptibility to induced DADs in HF myocytes by rapid pacing in the presence of 100 nM noradrenalin.

Figure 5A shows typical examples of APs and DAD of myocytes isolated from an ω3-PUFA and an ω9-MUFA fed HF animal. In the myocyte of the ω9-MUFA fed HF rabbit, but not in the myocyte of the ω3-PUFA fed HF rabbit, a DAD (arrow) was present. The amount of myocytes with more than one DAD was significantly lower ($P < 0.05$, Fisher's exact test) in ω3-PUFA fed HF rabbits compared to those of ω9-MUFA fed HF animal (**Table 3**). In addition, the number of DADs was significantly lower in the myocytes of ω3-PUFA fed HF rabbits (**Figure 5B**). **Figure 5C** summarizes the average AP characteristics at 3 Hz of myocytes isolated from ω3-PUFA and an ω9-MUFA fed HF animal in the presence of 100 nM noradrenaline. In presences of 100 nM noradrenaline, no AP differences were observed between myocytes of ω3-PUFA and an ω9-MUFA fed HF animal.

HYPERTROPHY IS SIMILAR IN ω3-PUFAs AND ω9-MUFAs FED RABBITS

Various studies demonstrate that ω3-PUFAs suppress development of hypertrophy and HF (Takahashi et al., 2005; Duda et al.,

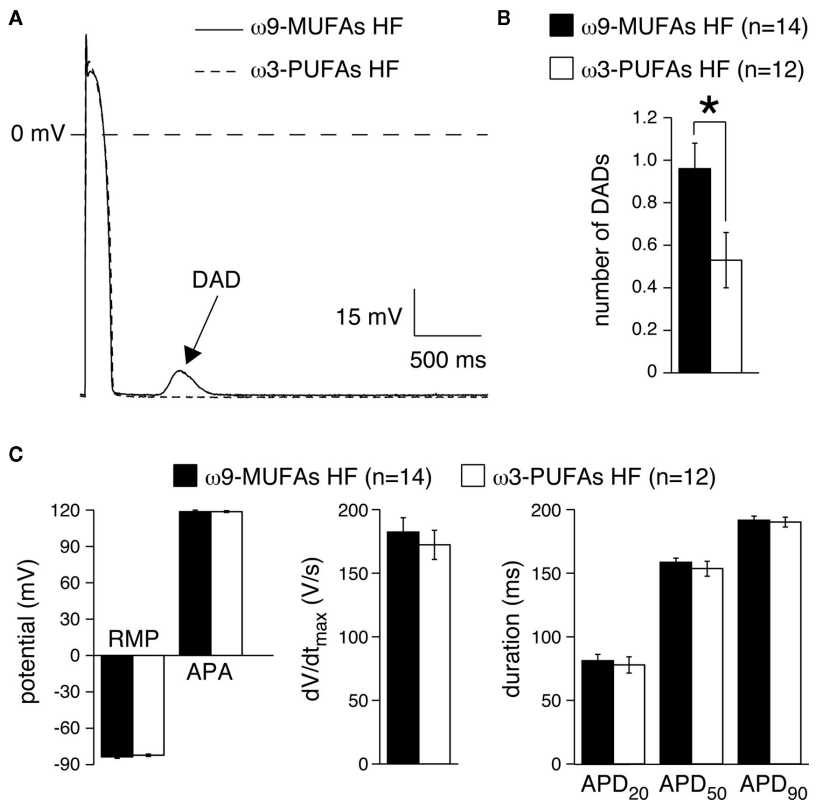

FIGURE 5 | (A) Typical examples of action potentials (AP) and delayed afterdepolarizations (DADs, arrow). **(B)** Average number of DADs, **(C)** Average AP characteristics. RMP, resting membrane potential; APA, AP amplitude; dV/dt_{max}, maximal AP upstroke velocity; APD_{20}, APD_{50}, and, APD_{90}, AP duration at 20, 50, and 90% repolarization, respectively, $^*P < 0.05$.

2007; Ramadeen et al., 2010; Chen et al., 2011). Because cardiac hypertrophy leads to a decrease of the surface to volume ratio of myocytes, an increased number of NHE-1 proteins are required to maintain a normal "cytoplasmic" NHE-1 mediated acid load recovery. The maintained cytoplasmic NHE-1 activity observed in cardiomyocytes from ω3-PUFAs treated HF rabbits (**Figure 4**) may thus be explained also by a lower degree of hypertrophy. Therefore, we finally analyzed important parameters for hypertrophy and HF in ω3-PUFA and ω9-MUFA fed rabbits, which underwent volume- and pressure-overload. Body, lung, and heart weight were similar in ω3-PUFA and ω9-MUFA rabbits (**Figure 6A**). Consequently, relative lung weight and relative heart weight, an index of cardiac hypertrophy, were not significantly different (**Figure 6B**). While previously we observed relative heart weights of 2.2–2.5 in non-failing rabbits with a standard chow diet (Baartscheer et al., 2003a,b, 2005, 2008; de Groot et al., 2003; van Borren et al., 2006), the relative heart weights in the present study of ω3-PUFA and ω9-MUFA fed rabbits were 3.6 ± 0.26 and 3.3 ± 1.13, respectively. Thus, despite the absence of differences in degree of hypertrophy, both ω3-PUFA and ω9-MUFA fed HF rabbit hearts are equally hypertrophied. Moreover, cell capacitance, an electrophysiological measure of cell size, was not significantly different between myocytes of ω3-PUFA and ω9-MUFA rabbits (**Figure 6C**). Furthermore, the presence of ascites assessed at autopsy was the same in ω3-PUFA and ω9-MUFA rabbits. These data indicate that the degree of hypertrophy and HF are similar in ω3-PUFA and ω9-MUFA fed rabbits.

Table 3 | Susceptibility to induce delayed afterdepolarizations (DADs).

	Fewer than 1 DAD	1 or more DAD
ω9-MUFA	2	12
ω3-PUFA	7	5

Number of myocytes. Values indicate the number of myocytes having <1 or ≥1 DAD (average of five tracings). The susceptibility to induce DADs is significantly lower in ω3-PUFA compared to ω9-MUFA myocytes ($P < 0.05$, Fisher's exact test).

DISCUSSION

OVERVIEW

In this study we examined the effects of dietary ω3-PUFAs on NHE-1 of myocytes from healthy and failing hearts. In general, NHE-1 inhibition is thought to be a pharmacological tool for the treatment of various detrimental cardiac conditions such as ischemia/reperfusion injury, arrhythmias, hypertrophy, and HF. In many pre-clinical studies, NHE-1 inhibition has been shown to reduce ischemia/reperfusion injury (Lee et al., 2005; Ayoub et al., 2010). In clinical trials, however, the cardioprotective effects of NHE-1 inhibition were less clear and the treatment with the NHE-1 inhibitor cariporide was associated with significantly greater incidence of stroke (Fliegel and Karmazyn, 2004). These adverse effects halted the further use of cariporide as cardioprotective agent.

Cardiac NHE-1 activity is also significantly increased in animal and patients with HF (Baartscheer et al., 2003a; Chahine et al., 2005). Pre-clinical studies demonstrated that chronic inhibition of NHE-1 leads to reversal of cardiac fibrosis, hypertrophy and HF, and improved contractility in HF models in mice (Engelhardt et al., 2002), rats (Camillión de Hurtado et al., 2002; Chen et al., 2004), and rabbit (Baartscheer et al., 2005, 2008). In rabbit studies, a diet containing the NHE-1 inhibitor cariporide not only reversed hypertrophy and reduced signs of HF, but also reversed cardiac ionic and electrical remodeling and prevented changes in myocyte dimensions, AP duration, and NHE-1 fluxes (Baartscheer et al., 2005, 2008). In addition, Ca^{2+} homeostasis remained undisturbed, and no increase of the incidence of Ca^{2+} after transient dependent DADs occurred (Baartscheer et al., 2005). From the prevention of excessive fibrosis, prolongation of AP duration, and DADs (Nuss et al., 1999; Marx et al., 2000; Sipido et al., 2000; Janse, 2004; Pogwizd and Bers, 2004), one may infer that NHE-1 inhibition is

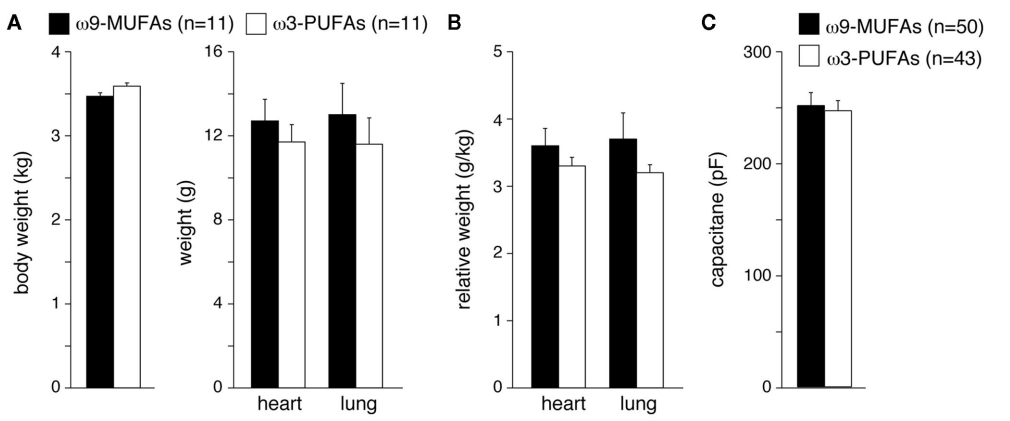

FIGURE 6 | (A) Average body, lung, and heart weight in ω3-PUFA and ω9-MUFA fed rabbits which underwent volume- and pressure-overload. *n*, Number of rabbits **(B)**, Relative lung weight and relative heart weight, an index of cardiac hypertrophy, in ω3-PUFA, and ω9-MUFA fed rabbits which underwent volume- and pressure-overload. **(C)** Average cell membrane capacitance of myocytes isolated from ω3-PUFA and ω9-MUFA rabbits. *n*, Number of myocytes. $*P < 0.05$.

also anti-arrhythmic. Thus, at least part of the beneficial effects of ω3-PUFAs may be attributed to NHE-1 inhibition during HF.

DIETARY ω3-PUFAs DO NOT SUPPRESS THE NHE-1 IN MYOCYTES OF HEALTHY ANIMALS

In myocytes isolated from healthy animals, we found that ω3-PUFAs do not affect the NHE-1 activity as compared to ω9-MUFAs (**Figure 2**). Our results contrast with a study by Goel et al. (2002) that showed that ω3-PUFAs reduced the NHE-1 activity. This discrepancy is likely due to study design. We studied dietary ω3-PUFAs intake that causes ω3-PUFA incorporation into cardiac cell membranes (Owen et al., 2004), whereas Goel et al. (2002) studied direct application of ω3-PUFAs on cardiac myocytes. Acutely applied ω3-PUFAs and incorporated ω3-PUFAs have different effects on cardiac electrophysiology (den Ruijter et al., 2007, 2010; Verkerk et al., 2009), and our study suggests that it also has a different effect on pH_i. Dietary ω3-PUFAs intake does not affect the resting pH_i in healthy animals (present study), while acute administration resulted in acidosis (Aires et al., 2003), which could well explain the lower apparent NHE-1 activity observed by Goel et al. Other explanations may be the differences in species (pig vs. rabbit) and the technique used to measure NHE-1 activity (radioactive Na^+ uptake in cell suspensions vs. single cell fluorescence).

DIETARY ω3-PUFAs SUPPRESS NHE-1 UPREGULATION IN A RABBIT MODEL OF VOLUME- AND PRESSURE-OVERLOAD

In our rabbit model of volume- and pressure-overload, we found that the NHE-1 activity in ω3-PUFAs myocytes was significantly lower than in ω9-MUFAs myocytes (**Figure 3**). The mechanisms for the lower NHE-1 activity are unknown. One may speculate that ω3-PUFAs affect membrane fluidity (Jahangiri et al., 2000), resulting in a decrease of NHE-1 activity (Bookstein et al., 1997). This membrane fluidity theory is frequently used to explain the effects of ω3-PUFAs on membrane channels, however, but the lack of significant effects of ω3-PUFAs on NHE-1 in healthy animals is not in line of this hypothesis.

A second hypothesis is that ω3-PUFAs attenuate cardiac hypertrophy (Takahashi et al., 2005; Duda et al., 2007; Ramadeen et al., 2010; Chen et al., 2011), resulting in a decrease of NHE-1 activity (Baartscheer et al., 2005). In the present study, however, the relative heart weight and cell capacitance, both indices of cardiac hypertrophy, did not differ between ω3-PUFAs and ω9-MUFAs fed rabbits or their myocytes, respectively (**Figure 6**). This excludes differences in hypertrophy as a likely mechanism. The unaltered degree of hypertrophy is in contrast with observations in mice with transverse aortic constriction (Chen et al., 2011) and juvenile visceral steatosis (Takahashi et al., 2005), rats with abdominal aortic banding (Duda et al., 2007), and rapid-paced dogs (Ramadeen et al., 2010) where dietary supplementation of ω3-PUFAs attenuated cardiac hypertrophy. This discrepancy may be explained by differences in HF model, species, ω3-PUFAs concentration, but also to the control diets used. In our study, the control (ω9-MUFAs) diet was supplemented with HOSF, while in the other studies the control diet was standard chow or supplemented with corn oil. The importance of a proper control diet is supported by the finding that the relative heart weight of ω9-MUFAs fed HF rabbits was ≈3.6 (**Figure 6**), while in the same rabbit HF model with a standard chow diet we previously measured relative heart weights of 4.4–6.0 in our laboratory (Baartscheer et al., 2003a,b, 2005, 2008; de Groot et al., 2003; van Borren et al., 2006; den Ruijter et al., 2008). This suggests that both ω3-PUFAs and ω9-MUFAs diets reduce the degree of hypertrophy. Further studies are required to address this.

A third hypothesis is that dietary ω3-PUFAs affect cell signaling and enzymes important for NHE-1 activity. The NHE is not only activated by pH_i but also by a number of other stimuli. NHE-1 activity is accelerated in response of endothelin, angiotensin II, and G protein and second messenger stimulation of PKC (diacylglycerol) and PKA (forskolin, β1-adrenoreceptor agonists; Kandasamy et al., 1995; Karmazyn et al., 2001; Díaz et al., 2010). Also, mitogen-activated protein (MAP) kinase-dependent pathways result in the phosphorylation of the NHE (Sartori et al., 1999). ω3-PUFAs affect several of these NHE-1 stimuli. They reduce diacylglycerol and PKC, activate the parasympathetic nervous system, and reduce angiotensin-converting enzyme (ACE) activity (Mohan and Das, 2001; Seung-Kim et al., 2001; Takahashi et al., 2005), although the latter is not a consistent finding (Ogawa et al., 2009). An intriguing question is why the NHE-1 activity in myocytes of ω3-PUFA fed animals is lower than in myocytes of ω9-MUFA fed animals in our model of volume- and pressure-overload, while it is not different in healthy rabbits. This suggests that the NHE-1 reduction is due to changes in cell signaling pathways active during HF (Onohara et al., 2006; Niizeki et al., 2008). One can speculate that the lower NHE-1 activity also reduces the degree of hypertrophy and HF, but this was not observed in the present study, suggesting that NHE-1 is more sensitive for ω3-PUFAs modulation of cellular signaling pathways than hypertrophy and HF. Alternatively, the time required for hypertrophy and HF attenuation might be longer than that for NHE-1 reduction. The decreased NHE-1 activity may also be the result of a decreased expression of NHE-1.

DIETARY ω3-PUFAs SUPPRESS DADs

In our HF model of volume-and pressure-overload, we observed that the susceptibility for DAD development was significantly lower in myocytes of ω3-PUFAs fed rabbits compared to those of ω9-MUFAs fed rabbits (**Table 3**; **Figure 6B**). DADs occur in $[Ca^{2+}]_i$ overload condition (Verkerk et al., 2000a, and primary references cited therein). Thus, our results indicate that myocytes of ω3-PUFAs fed failing rabbits are less sensitive for $[Ca^{2+}]_i$ overload development than those of ω 9-MUFAs fed failing rabbits. According to the importance of the NHE-1 for $[Ca^{2+}]_i$ (Baartscheer et al., 2003a), it is tempting to speculate that this is due to the lower NHE-1 activity in myocytes of ω3-PUFAs fed HF rabbits. Previously we observed multiple changes in ionic currents due to dietary and acute ω3-PUFAs resulting in AP shortening (Verkerk et al., 2006) and reduced susceptibility of early afterdepolarizations and DADs development (den Ruijter et al., 2006, 2008; Berecki et al., 2007).

Dietary ω3-PUFAs caused an increase of I_{K1} resulting in a more stable RMP (Verkerk et al., 2006). The latter will result in smaller DAD amplitudes. In addition, dietary ω3-PUFAs decreased I_{NCX} (Verkerk et al., 2006), which carries the transient inward current, I_{ti}, responsible for DADs (Verkerk et al., 2000a, and primary references cited therein). Decreased I_{NCX}

may therefore also result in DADs of smaller amplitude. However, while both changes may reduce the DAD amplitude, they will not reduce the propensity to spontaneous Ca^{2+} release of the SR and thus DADs. Previously, we observed AP shortening due to dietary ω3-PUFAs (Verkerk et al., 2006). AP shortening leads to an increased diastolic interval, favoring removal of excess Ca^{2+} from the cytosol. This will reduce $[Ca^{2+}]_i$ overload conditions and DAD occurrence (Verkerk et al., 2000b, and primary refs. cited therein). However, in presence of 100 nM noradrenaline, AP duration did not differed significantly between myocytes of ω3-PUFAs and ω9-MUFAs fed HF rabbits (**Figure 5C**). Thus, in the present study the role of AP duration in susceptibility of DADs is limited, although this cannot be entirely excluded in the absence of data on intracellular calcium and sodium activity.

CONCLUSION

Dietary ω3-PUFAs from FO suppress upregulation of the NHE-1 activity in a rabbit model of volume- and pressure-overload. The degree of hypertrophy and HF is similar in myocytes of ω3-PUFAs and ω9-MUFAs fed HF rabbits, but the lower NHE-1 activity in myocytes of ω3-PUFAs fed HF rabbits suggests that dietary ω3-PUFAs administration during the development of HF may be anti-arrhythmic via reduction of ischemia/reperfusion injury and Ca^{2+}-modulated arrhythmias.

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Omega 3 fatty acid inhibition of inflammatory cytokine-mediated Connexin43 regulation in the heart

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Background: The proinflammatory cytokine Interleukin-1β (IL-1β), which increases in the heart post myocardial infarction (MI), has been shown to cause loss of Connexin43 (Cx43) function, an event known to underlie formation of the arrhythmogenic substrate. Omega 3 Fatty acids exhibit antiarrhythmic properties and impact IL-1β signaling. We hypothesize that Omega-3 fatty acids prevent arrhythmias in part, by inhibiting IL-1β signaling thus maintaining functional Cx43 channels. **Methods:** Rat neonatal myocytes or Madin-Darby Canine Kidney Epithelial (MDCK) cells grown in media in the absence (Ctr) or presence of 30 μM docosahexaenoic acid (DHA, an Omega-3 Fatty acid) were treated with 0.1 μM activated IL-1β. We determined Cx43 channel function using a dye spread assay. Western blot and immunostaining were used to examine Cx43 levels/localization and downstream effectors of IL-1β. In addition we used a murine model of MI for 24 h to determine the impact of an Omega-3 fatty acid enriched diet on Cx43 levels/localization post MI. **Results:** IL-1β significantly inhibited Cx43 function in Ctr cells (200.9 ± 17.7 μm [Ctr] vs. 112.8 ± 14.9 μm [0.1 uM IL-1β], $p < 0.05$). However, DHA-treated cells remained highly coupled in the presence of IL-1β [167.9 ± 21.9 μm [DHA] vs. 164.4 ± 22.3 μm [DHA + 0.1 uM IL-1β], $p < 0.05$, $n = 4$]. Additionally, western blot showed that IL-1β treatment caused a 38.5% downregulation of Cx43 [1.00 au [Ctr] vs. 0.615 au (0.1 μM IL-1β) which was completely abolished in DHA-treated cells (0.935 au [DHA] vs. 1.02 au [DHA + 0.1 μM IL-1β), $p < 0.05$, $n = 3$]. Examination of the downstream modulator of IL-1β, NFκβ showed that while hypoxia caused translocation of NFκβ to the nucleus, this was inhibited by DHA. Additionally we found that a diet enriched in Omega-3 Fatty acids inhibited lateralization of Cx43 in the post-MI murine heart as well as limited activation of fibroblasts which would lead to decreased fibrosis overall. **Conclusions:** Omega 3 Fatty acid treatment inhibited IL-1β-stimulated loss of Cx43 protein, and more importantly, inhibited loss of Cx43 function by inhibiting translocation of NFκβ. In the intact heart a diet enriched in Omega 3 Fatty Acids limited loss of Cx43 at the intercalated disk in the heart following MI. These data suggest that one of cardio-protective mechanisms by which Omega 3 Fatty acids work includes prevention of the pro-arrhythmic loss of Cx43 post MI and the attenuation of cardiac fibrosis after injury.

Keywords: arrhythmia, fibrosis, gap junction, inflammation, interleukin, myocardial infarction

INTRODUCTION

Gap junctions provide direct electrical continuity between myocytes in the functioning myocardium. These junctions are formed from connexin proteins which are four transmembrane domain proteins which oligomerize form a half channel known as a connexon. Connexons from apposing cells meet head-to-head across the extracellular space and form a full channel that allows for direct cytoplasmic continuity between the cells. In the heart this channel is a low resistance pore which allows for rapid electrical conduction through the working myocardium. Loss of gap junctions in cardiac injury is associated with increased arrhythmogenicity and Sudden Cardiac Death (Peters et al., 1997; Gutstein et al., 2001).

In 1978, Dyerberg et al. reported that the Greenland Inuit population had higher than average levels of the long chain *n-3* fatty acid (*n-3* LCFA now known as Omega 3 Fatty Acids or w3 fatty acids) and importantly they had an associated decreased prevalence of atherosclerotic disease (Dyerberg et al., 1978). This report set off decades of research into the mechanisms by which these LCFA might work and what the extent of their cardiovascular benefits might be. A recent meta analysis showed that dietary intake of w3 fatty acids is associated with a significant decrease in sudden cardiac death (35.1% decrease) (Musa-Veloso et al., 2011). While the understanding of the benefits of dietary intake of w3 fatty acids has come a long way, the molecular mechanisms by which these work are less understood. Although there have been several studies examining the molecular mechanisms by which w3 fatty acids alter cellular behavior it is still unclear the extent to which fatty acids directly alters cellular functions involved in cardiac electrophysiology and how they work to do so. Being as gap junctional conduction is a key player in normal cardiac conduction, in this study we examined the effects of w3 fatty acids on the

levels, localization, and function of the primary ventricular gap junction protein Cx43.

The initial thought on how w3 fatty acids worked was that it intercalated itself into the cellular membranes thereby altering the fluidity of the membranes which then, in turn, altered ion channel behavior (Hallaq et al., 1990; Macleod et al., 1998; Leaf et al., 2002). More recent studies have shown that w3 fatty acids alter gene expression via activation of nuclear factor kappa beta (NFκβ) as well as direct interaction with transcription factors in the nucleus (Di Nunzio et al., 2009). Being as NFκβ alters the response of myocytes to the inflammatory cytokine Interleukin 1β, which is upregulated following myocardial infarction (MI) (Abbate et al., 2010), and we have shown that IL-1β affects the gap junction protein Connexin43 (Cx43) in both the nervous system (Duffy et al., 2000) in the injured heart (Baum et al., 2012), we hypothesized that one of the mechanisms for the anti-arrhythmic effects of a diet containing w3 fatty acids is by regulating Cx43 containing gap junctions following myocardial injury.

METHODS

MOUSE DIETS

All animal procedures were done with approval from the Animal Care Institute (IACUC) at Beth Israel Deaconess Medical Center and in compliance with NIH guidelines. C57 black mice were fed *ad libitum* diets either enriched for Omega 3 Fatty Acids (3% of total calories) or a matched diet of 3% lard (Harlan, Madison, WI) for 6 weeks starting from the age of 6 weeks. At the end of the diet period the mice from each diet group were split into control or MI groups. Both groups of mice were anesthetized and a small incision was made in the left thorax. Control mice were closed and allowed to recover for 24 h. MI mice had the left anterior descending coronary artery ligated at the place where the artery surfaced on the front of the heart. These mice were closed and allowed to recover for 24 h. After 24 h mice from all groups were sacrificed and hearts were excised for biochemical studies.

DYE SPREAD ASSAY

Cell cultures-MDCK cells were grown in 35 mm dishes in a medium of DMEM (ATCC) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin-streptomycin (Cellgro) then incubated overnight in 1 μM interleukin-1β (Sigma) with or without 3 mM DHA. After incubation media was removed and cells were scraped with a razorblade and incubated for 5 min in 0.5% Rhodamine Dextran plus 2.5% Lucifer Yellow in 150 mM Lithium Chloride. Following PBS rinses (3 × 10 min) cells were fixed in 4% formaldehyde for 15 min then examined on a Leica 5500 inverted microscope. Images were taken from three areas (at center, and at 25% from both top, and bottom of image) of five separate dishes per treatment and dye spread was measured from the scrape line to the furthest edge of dye spread (Image J NIH Shareware). Statistical analysis was done using an ANOVA with Bonferroni correction.

CELL CULTURE

MDCK cells were plated either in 35 mm dishes or on glass cover slips for Western blot and Immunohistochemistry, respectively. Cells were maintained in DMEM + 10% Fetal bovine serum and 0.01% PenStrep until confluent. Cells were then treated with 0.01 mM IL-1β overnight then fixed in 4% formaldehyde and stained for Cx43 as described below.

WESTERN BLOT

Cell cultures and tissue samples were lysed in complete lysis buffer (50 mmol/L Tris-HCl pH 7.4, 0.25 mmol/L Na-deoxycholate, 150 mmol/L NaCl, 2mM EGTA, 0.1 mmol/L Na3VO4, 10 mmol/L NaF, 1 mmol/L PMSF, 1% Triton-X 100, ½tablet of Complete Protease Inhibitor (Roche Biochemicals, Indianapolis, IN). Lysates were sonicated for 30 s, maintained on ice for 30 min. then triturated and spun at 10,000 rpm for 10 min. Following removal of the pellet protein levels were tested using BCA protein Assay Kit (BioRad). Matched levels of total protein were mixed with loading buffer (2X laemini buffer + DTT), then run for Western blots using 10% SDS-PAGE gels. Gels were Commassie blue stained as loading control (Sohlenius et al., 1996) and then proteins were transferred to nitrocellulose membranes and probed for Cx43 (Sigma). Bands were analyzed by densitometry (Cx43/Commassie blue) using NIH Scion Image.

IMMUNOHISTOCHEMISTRY

Rapidly-frozen heart samples from fixed cell cultures or from epicardium of mouse hearts were sectioned (15 microns) using a Leica 3050S cryostat. Sections were fixed in 4% formaldehyde for 30 min at RT then incubated in 50 mM NH_4Cl for 30 min to quench autofluorescence. Following quench, sections were blocked (PBS + 10% goat Serum + 0.4% Triton-X 100) for 1 h at RT then incubated with primary antibodies directed against Cx43 (Sigma) at 4°C overnight. Following 30 min rinse (3 × 10 min, PBS + 0.4% Triton-X 100) slices were incubated with secondary antibodies (Alexa Fluor, anti-mouse 488 and anti-rabbit 595) for 1 h at RT. Slices were rinsed for 50 min (5 × 10 min), and mounted on glass microscope slides with Vectashield anti-fade agent (Vector Laboratories, Burlingame, CA) and examined using a Zeiss Axiophott 200 M equipped with both FITC and Texas Red filters.

Epicardial mapping-Mice ($n = 3$ per group) were anesthetized with 5% vaporized isoflurane and maintained at 2–3% vaporized flow. The chest was opened and an electrode array (10 × 6 mm) was placed on the left ventricle and electrical signals were collected using a UnEmap system (Aukland, New Zealand). This system amplifies, records, stores and analyzes time of occurrence of electrical signals, and graphically displays the data as activation maps. Rates of inducibility into VT were compared by ANOVA and considered to be significantly different at $p < 0.05$.

RESULTS

OMEGA 3 FATTY ACIDS LIMIT IL-1 BETA-INDUCED LOSS OF GAP JUNCTION FUNCTION

To determine the effects of w3 fatty acids on gap junction function we used a standard scrape-loading method to examine Cx43 channel function (**Figure 1**). Cells were incubated with IL-1β or IL-1β plus DHA and the distance that Lucifer Yellow dye spread was measured and used as an indicator of gap junction

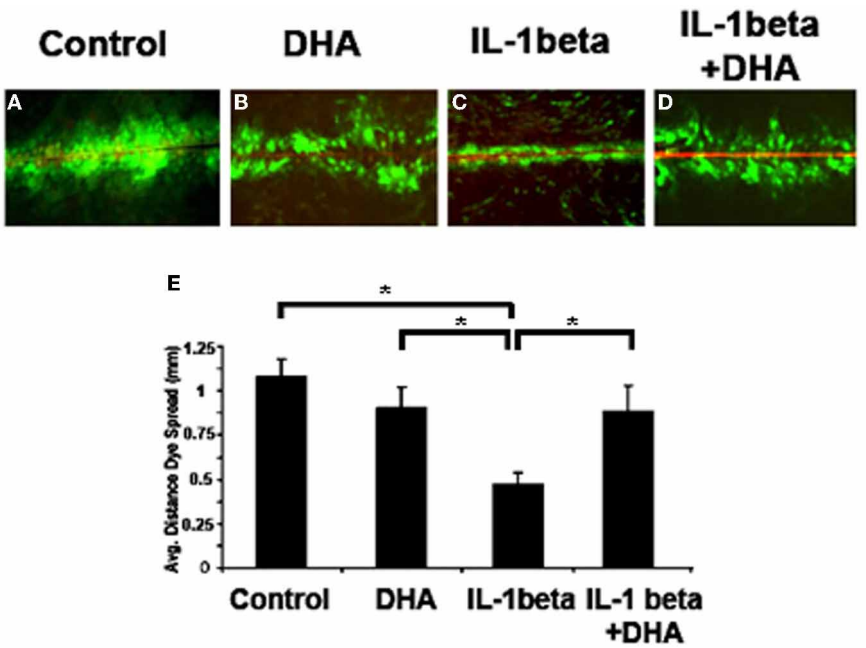

FIGURE 1 | Dye spread in MDCK cells. Lucifer Yellow (LY, green) easily passed through gap junctions in control cells **(A)**, an event not altered by DHA treatment alone **(B)**. In contrast IL-1β treatment caused a significant decrease in coupling **(C)**which was inhibited in the presence of DHA **(D)**. Quantification of dye spread is seen in **E**. Texas Red Dextran (red) was used to mark broken cells. Cells with red in them were not included in the analysis. $N = 3$, $p < 0.05$.

function. Control cells passed Lucifer Yellow (LY) though gap junctions more than 1.0 mm on average (**Figure 1A**). Incubation of control cells with DHA alone had no significant effect on LY transfer. In contrast, incubation of cells with IL-1β significantly decreased dye spread to 0.48 mm showing a loss of gap junctional function. When cells were pretreated with DHA then incubated with IL-1β, channel function was maintained at levels comparable to normal despite the presence of IL-1β. These data indicate that w3 fatty acids inhibit the IL-1β-induced loss of Cx43 function.

EFFECTS OF DHA ON Cx43 LEVELS

To assess whether DHA changed the total levels of Cx43 in cardiac myocytes, either at the cell membrane or within the cytoplasm of the cell, we performed Western-blotting of total, soluble (cytoplasmic) and insoluble (membrane) fractions of neonatal rat ventricular myocytes (**Figure 2**). Examination of total levels of Cx43 showed that IL-1β decreased Cx43. DHA treatment alone had no effect on control cells but inhibited the loss of Cx43 in the presence of IL-1β (**Figure 2A**). To determine if DHA was affecting the membrane localized Cx43 preferentially, which would suggest that the mechanism of increased Cx43 was via membrane stabilization, we examined Cx43 levels in membrane (**Figure 2B**, Insoluble Cx43) vs. cytosolic (**Figure 2C**, Soluble Cx43) fractions of control, DHA alone, IL-1β and IL-β + DHA treated cardiac myocytes. We found that IL-1β did not significantly decrease the amount of Cx43 found in the insoluble membrane fraction of the cells. Instead the decrease in Cx43 was seen only in the cytosolic fraction suggesting that IL-1β induced loss of Cx43 is due to changes in the production of Cx43 rather than in its stabilization at the cell membrane. Pretreatment with DHA significantly increased both the insoluble membrane form of Cx43 showing that it has some affects on stabilization of Cx43 within cell membranes but in addition it also increased the soluble cytosolic form of Cx43 suggesting it was also impacting gene expression and/or protein degradation of Cx43. These data indicate that the maintenance of gap junction function seen with DHA treatment may be via two pathways, one being increased open channels in the cellular membranes and the second being from increased expression of Cx43.

NFκβ TRANSLOCATION IS INHIBITED BY DHA

NFκβ is a downstream mediator of IL-1β signaling which has been shown to bind to the Cx43 promotor and regulate Cx43 levels (Alonso et al., 2010). Therefore, to determine if the mechanism by which DHA inhibits IL-1β-induced loss of Cx43 was via this canonical IL-1β signaling pathway we examined the effect of DHA on NFκβ activation by immunostaining for the active p65 form of NFκβ (**Figure 3**) in NRVMs. Control cells showed low levels of p65 NFκβ (**Figure 3A**) but IL-1β treatment of NRVM caused a significant increase in active p65 NFκβ (**Figure 3B**, $p < 0.05$). DHA alone had no effect on p65 NFκβ levels (**Figure 3C**). In contrast to the IL-1β treated cells, cells incubated with DHA and IL-1β had p65 NFκβ levels that were not significantly different from control cells (**Figure 3D**). This suggests that one mechanism of action of w3 fatty acids may be by blocking p65 NFκβ activation thereby inhibiting the IL-1β signaling cascade (**Figure 3**).

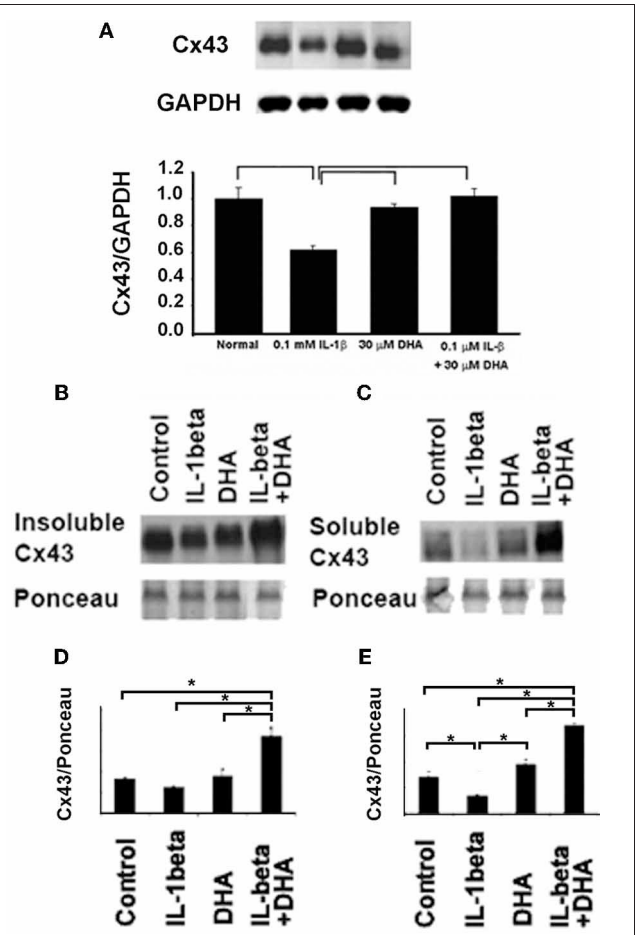

FIGURE 2 | Western-blotting results for the effect of DHA treatment on changes in total (A) membrane (B), and cytoplasmic (C) Cx43 in neonatal rat ventricular myocytes (NRVM). IL-1β application led to a significant loss of total Cx43 **(A)**. This loss was seen in both soluble and insoluble fractions (**B** and **C**). DHA inhibited this effect. Quantification of the insoluble **(D)** and soluble **(E)** Cx43 fractions is shown below. Ponceau staining was used as a loading control. Densitometry of the bands ($n = 3$) is shown $p < 0.05$.

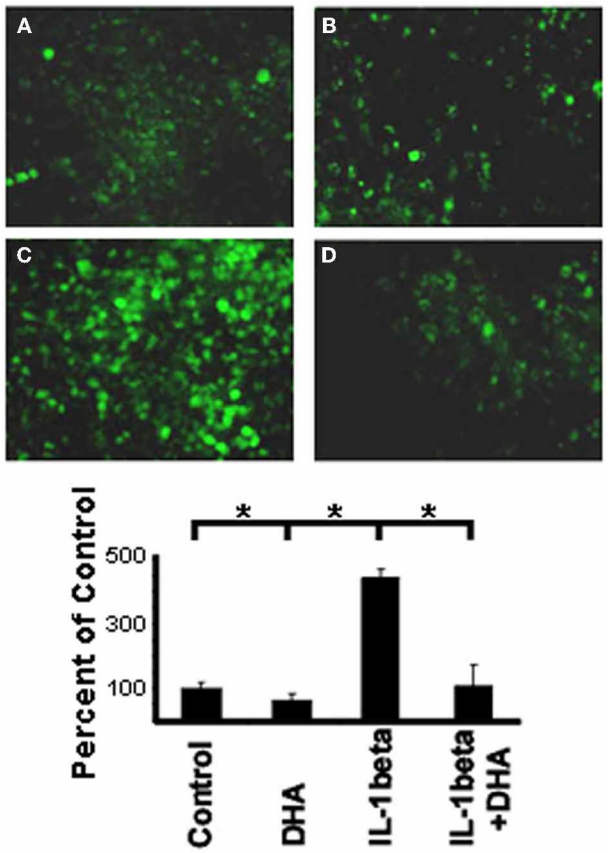

FIGURE 3 | Immunofluorescence of activated p65 NFκB in NRVM. Control cells show low levels of NFκB staining **(A)** which is not change with DHA incubation alone **(B)**. In contrast, stimulation with IL-1β led to significant increases in NFκB activation **(C)**. DHA incubation inhibited activation of NFκB back to control levels **(D)**. ($n = 3$, $p < 0.05$).

EFFECTS OF DHA *In vivo*

Our previous experiments showed that DHA normalizes Cx43 under inflammatory conditions in cell culture. To determine the effects of DHA *in vivo* we pretreated mice with two different diets for six weeks. The first group of mice received a DHA enriched diet (2.5%) while control mice were fed a matched fat diet (2.5% lard/corn oil). We then ligated the left anterior descending coronary artery (LAD), waited for 24 h and then harvested the hearts. **Figure 4** shows the results of immunostaining from control and DHA pretreated mouse hearts. Cx43 can be seen in red, while Cadherin (used to mark intercalated disks) is in green. Increased lateralization of Cx43 can be seen in control mice following coronary occlusion (CO). However, DHA pretreatment seemed to completely reverse this lateralization. This suggests that DHA pretreatment may be able to prevent Cx43 displacement and ensure the continued maintenance of normal gap junction localization following MI.

IMPACT OF DHA ON FIBROBLAST TO MYOFIBROBLAST TRANSFORMATION

Our previous work has shown that fibroblast to myofibroblast transformation following CO greatly affects normal Cx43 distribution in cardiac myocytes (Baum et al., 2012). We therefore decided to examine whether or not DHA could prevent myofibroblast activation *in vivo* and thereby maintain normal Cx43 localization post-MI. To answer this, we used our mouse diet model and then sectioned and immunostained the hearts for the presence of Smooth Muscle Actin, a marker of myofibroblasts (activated fibroblasts). **Figure 5** shows that normal mouse hearts (**Figure 5A**) or hearts from mice that ate the DHA enriched diet but did not have their coronary artery ligated (**Figure 5B**) have little or no myofibroblasts but that MI induces the activation of fibroblasts in the injured region (**Figure 5C**). In contrast, mice fed a DHA enriched diet showed a significant decrease in SMA staining following MI as compared with control or DHA alone mice (**Figure 5D**). Quantification of the SMA staining in heart sections is shown in **Figure 5E**. This data show that DHA inhibits fibroblast to myofibroblast transformation post MI. Further studies to examine the overall levels of fibrosis in these hearts are needed

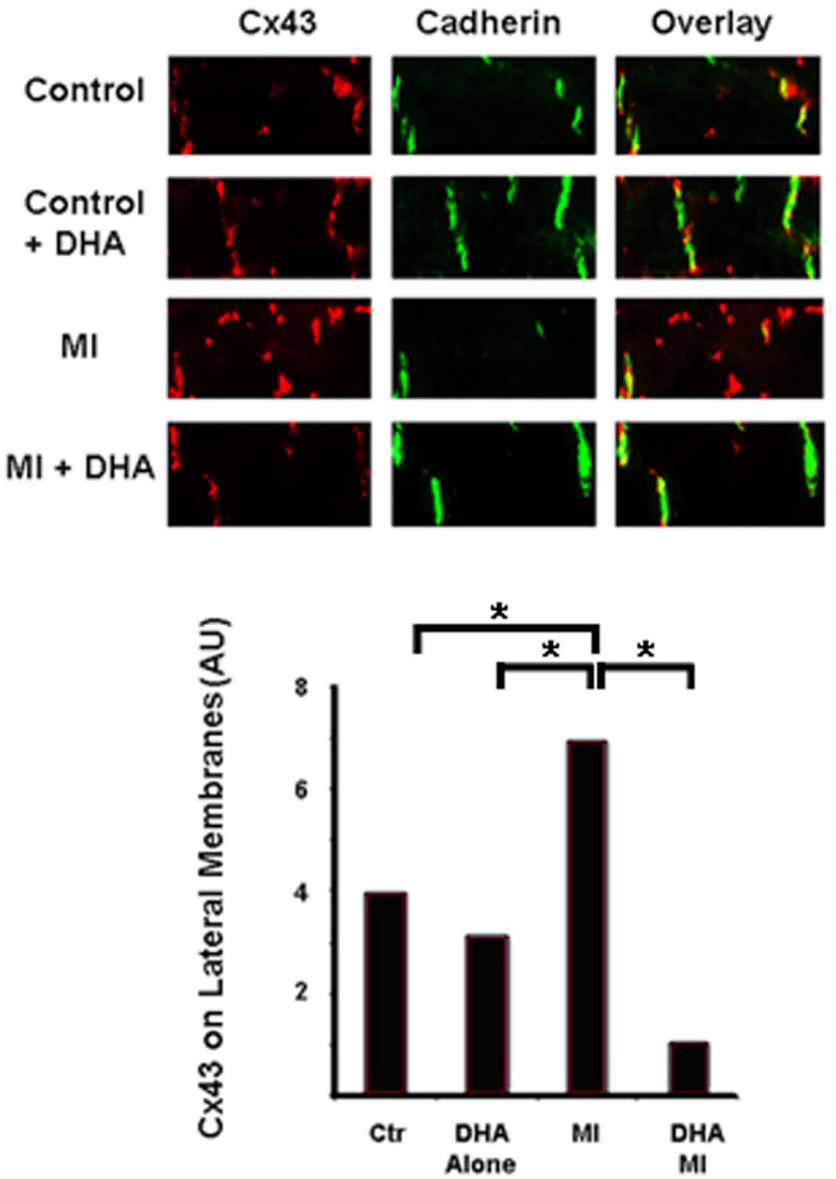

FIGURE 4 | Immunostaining of Cx43 distribution in control and DHA-treated mice following myocardial infarction. Cx43 (red) is found at the intercalated disks (Cadherin was used as a marker of the disk, green) in control animals in both diet groups (Control and Control + DHA). As expected MI caused lateralization of Cx43 (MI). This lateralization was completely inhibited in animals fed the DHA enriched diet (MI + DHA). $n = 3$, $p < 0.05$.

to determine if blockade of fibroblast transformation could help decrease fibrosis in the injured heart.

EFFECT OF A DHA ENRICHED DIET ON ARRHYTHMIA INDUCIBILITY IN THE INTACT HEART

To determine if chronic ingestion of a diet rich in w3 fatty acids was able to limit arrhythmogenicity in a model of MI animals from all groups were subjected to epicardial mapping for ventricular tachycardial (VT) inducibility. We found that control animals were unable to be stimulated into VT but while animals which ate a normal diet had normal sinus rhythm, they were easily induced into VT (**Figure 6A** and upper trace in **B**). In contrast, mice fed a diet enriched in w3 fatty acids were unable to be stimulated into VT (**Figure 6B**, lower trace). These data suggest that a diet enriched in DHA but eaten prior to any cardiac injury may have antiarrhythmic effects following MI.

DISCUSSION

Inflammatory processes in the heart lead to loss of Cx43 thus therapeutically targeting inflammation may decrease this loss and limit formation of arrhythmias. Studies have shown that diets enriched in w3 fatty acids lead to an attenuated inflammatory response by limiting the activation of the IL-1β receptor and limiting IL-1β cellular signaling through its nuclear mediator,

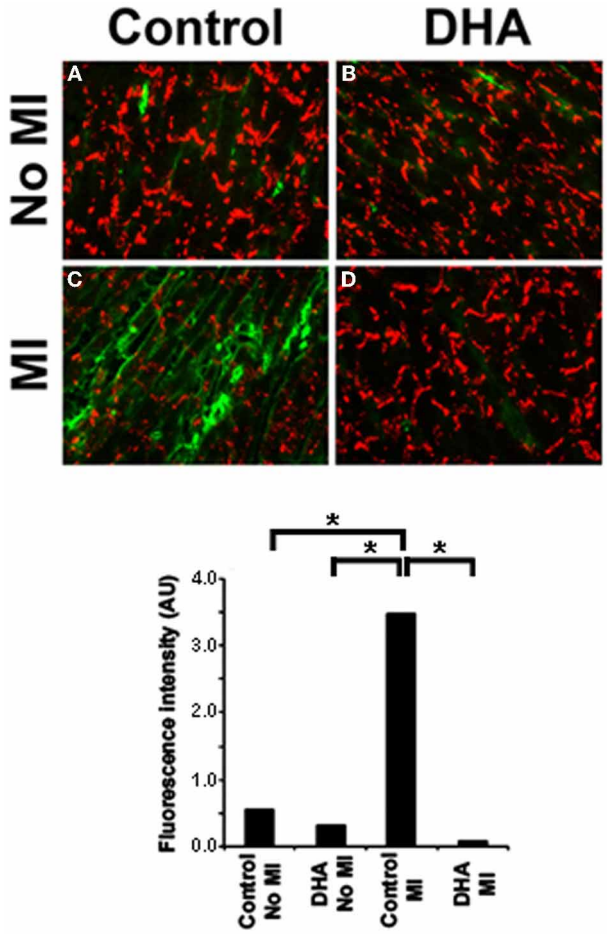

FIGURE 5 | Immunostaining for Cx43 (red) and SMA (green) in mice following coronary occlusion (CO) that received either a DHA or matched fat diet (Control). Control animals showed only very low levels of Smooth Muscle Actin staining regardless of their diet (**A** and **B**). In MI animals without DHA the number of SMA positive cells significantly increased after CO (**C**, MI). DHA pretreatment caused SMA positive staining to be restricted to vessels **(D)**. Lower panel shows the quantification of SMA staining intensity. $n = 3$, $p < 0.05$.

NFkB (Adkins and Kelley, 2010). In this study we examined the changes in NFkB following IL-1β stimulation with and without DHA, the w3 fatty acids and found that w3 fatty acids decreased NFkB translocation to the nucleus. Additionally, the loss of cellular coupling seen with IL-1β treatment was abrogated in the presence of DHA. Additionally total levels of Cx43 in the cellular membranes was increased in the presence of DHA which is likely due to stabilization of Cx within the membrane by the intercalation of the into the cellular membrane (Adkins and Kelley, 2010). Thus, DHA appears to affect coupling at two levels. First, DHA treatment appears to limit IL-1β signaling which has been shown to decrease Cx43 levels and function. Additionally DHA increased Cx43 within cellular membranes, helping to maintain coupling. Maintenance of cellular coupling throughout the ventricle is a requirement for normal electrical conduction through the heart. Studies have shown that heterogeneous loss of coupling increases ventricular arrhythmias by promoting regions of slowed conduction intermixed with regions of normal conduction. These regions of slowed conduction lead to formation of reverse conduction as electrical propagation from normal areas enters non-repolarizing regions ahead of the slow wave front. The figure of eight re-entrant circuits that are set up by this abnormal conduction pattern can then anchor spiral waves and lead ventricular tachycardia and fibrillation. We found mice fed high DHA diets were not inducible into VT under any circumstance. Thus, the changes we found in Cx43 and myofibroblast activation appeared to be at least part of a stabilizing substrate in these animals thus limiting the arrhythmogenicity of their hearts. It is of interest to note that these studies show that a diet given prior to the cardiac injury is capable of preventing subsequent damage after cardiac injury suggesting that the mechanism of action is to stabilize the healthy myocardium rather than to reverse remodel the injured myocardium.

One of the hallmarks of myocardial injury is the presence of Cx43 on the lateral membranes of cardiac myocytes as opposed to their normal localization at the intercalated disk (Peters et al., 1997). The initial thought was that these channels allowed for transverse conduction leading to slowing of conduction in the longitudinal direction and subsequent reentrant arrhythmias. Subsequent studies showed that the channels on the lateral membranes were non-functional (Yao et al., 2003) and that the anisotropic ratio, which would be expected to decrease if transverse conduction were increased, actually was larger in injured myocardium. Attempts have been made to identify the mechanisms by which lateralization occurs (Kieken et al., 2009) as well as identify therapies which limit loss of gap junctional coupling (Kjolbye et al., 2008; Wiegerinck et al., 2009). To date, therapies which decrease lateralization have not been found (Macia et al., 2011). In this study we have identified not a therapy *per se*, but a method by which lateralization of Cx43 can be limited following MI. We found that mice who had a prior exposure to a diet enriched in w3 fatty acids limited lateralization of Cx43 following MI. This decrease in lateralization was associated with maintenance of Cx43 at the intercalated disk so the w3 fatty acid was not just altering internalization of Cx43. In addition, examination of smooth muscle actin levels in the w3 fatty acids fed mice showed a marked decrease in the level of fibroblast activation. Based on our data showing w3 fatty acids decrease IL-1β signaling through NFκβ we hypothesize that the diet decreased the inflammatory processes in the heart thus limiting fibroblast activation.

In addition, following MI or other heart injury inflammatory processes begin the healing process which, when uncontrolled, leads to fibrosis which also contributes to formation of the arrhythmogenic substrate. Immune cells such as monocytes and macrophages migrate to the site of injury where they release pro-inflammatory cytokines. These cytokines affect a wide range of cellular processes and contribute to the transformation of resident fibroblasts to the activated myofibroblast phenotype. It is these myofibroblasts which then, through secretion of extracellular matrix components and regulators, lay down the collagenous bundles seen in fibrosis. In addition, during active healing these cells continue to produce such cytokines as TGF-β, IL-6, and

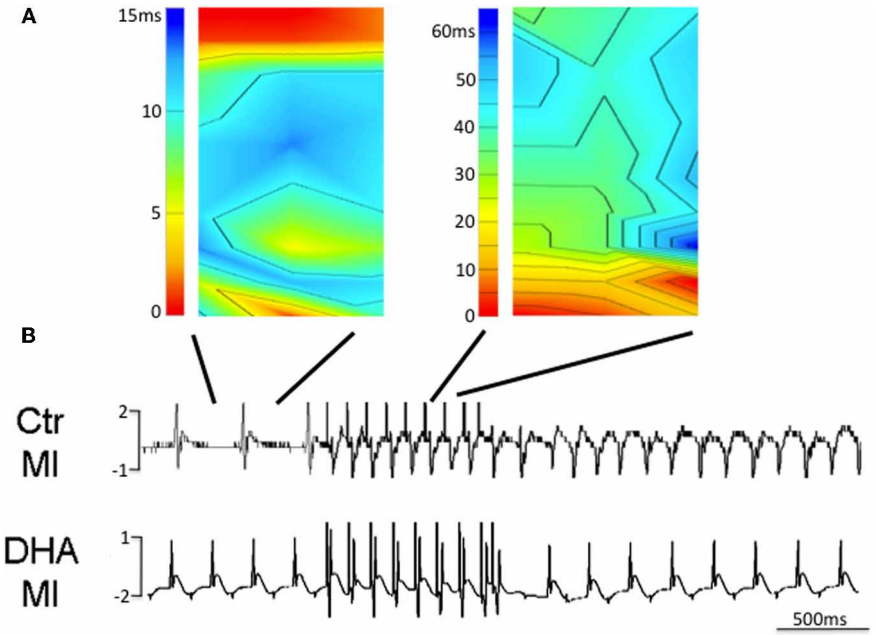

FIGURE 6 | Isochronal maps of sinus rhythm (SR) and induced ventricular tachycardia (VT) with a single premature stimulus (120 ms drive cycle, S2 60 ms) in a mouse fed a normal diet after MI (A and B upper trace, *n* = 3). In contrast, mice fed diets enriched in DHA were unable to be induced to VT with the same prematurity (**B**, lower trace, *n* = 3).

important for this discussion, IL-1β. Our previous studies have shown that IL-1β causes a decrease in Cx43 based cellular coupling in both the nervous system (Duffy et al., 2000) and the heart (Baum et al., 2012). In the heart the presence of myofibroblasts in injured regions leads to heterogeneous loss of Cx43 and formation of an arrhythmogenic substrate. The present studies show that a diet enriched in w3 fatty acids may limit activation of fibroblasts suggesting that it may decrease post-MI fibrosis. This decrease, combined with maintenance of functional gap junctions in the injured heart may be why w3 fatty acids may exhibit anti-arrhythmic characteristics.

In conclusion, our studies show that a regular diet which contains w3 fatty acids prior to any cardiac injury limit the loss of Cx43 induced by IL-β signaling through NFκβ. This protection from the loss of coupling is likely to cause a decrease in the arrhythmogenic potential of the heart. This could explain why studies have shown that almost half the reduction of risk for people on w3 fatty acids supplementation was due to a decrease in arrhythmic events and Sudden Cardiac Death (Levantesi et al., 2010). These data suggest that early and consistent dietary supplementation with w3 fatty acids may limit cardiovascular risk overall when part of the normal diet prior to any cardiac injury.

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