The Electrophysiologic Basis for the Antiarrhythmic Effect of n-3 Polyunsaturated Fatty Acids

Alexander Leaf

Departments of Medicine, Massachusetts General Hospital and the Harvard Medical School, Boston, MA, U.S.A.

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Send correspondence to:

Alexander Leaf, M.D.

Massachusetts General Hospital

Bldg.149, Rm 4001, 13th Street

Charlestown, MA 02129

USA

Tel: 617-726-5908

Fax: 617-726-6144

Aleaf@partners.org

Summary - Studies on the antiarrhythmic actions of n-3 polyunsaturated fatty acids in fish oils (PUFA) are reported. The PUFA stabilize the electrical activity of isolated cardiac myocytes by modulating sarcolemmal ion channels, so that a stronger electrical stimulus is required to elicit an action potential and the relative refractory period is markedly prolonged. Inhibition of voltage dependent sodium currents which initiate action potentials in excitable tissues, and also inhibition of the L-type calcium currents, which initiate release of sarcoplasmic calcium stores, that increase cytosolic free calcium concentrations and activate the contractile proteins in myocytes, appear at present to be the probable major antiarrhythmic mechanism of the PUFAs

It was the findings of McLennan and Charnock [1], which first conclusively demonstrated in rats that the n-3 fatty acids in fish oils prevented ischemia-induced fatal ventricular arrhythmias. Our studies sought to learn if we could confirm their findings in a reliable dog model of sudden cardiac death with Prof. George E. Billman. A surgically induced myocardial infarction was produced by ligating the left anterior decending coronary artery and an inflatable cuff was placed around the left circumflex artery. The dogs were allowed about a month to recover from the surgery and their myocardial infarction during which they were trained to run on a treadmill. The animals were then screened for susceptibility to fatal ventricular arrhythmias (VF) when their left circumflex artery was occluded while they were running on a treadmill. Some 60% of animals [2] were found susceptible and these were the dogs studied. Once an animal is "susceptible" it remains susceptible on further exercise-ischemia trials. In 10 of the 13 such dogs intravenous infusion of an emulsion of a concentrate of the fish oil free fatty acids (PUFAs) just prior to the exercise-ischemia test prevented the fatal VF ($p<0.005$) [3]. In the control exercise-ischemia tests one week prior to the test with the infusion of the (PUFAs) and one week following that test all animals developed ischemia-induced VF requiring prompt defibrillation. In additional studies [4] we have found that pure eicosapentaenoic acid C20:5n-3, EPA) or docosahexaenoic acid (C22:6n-3, DHA), or α-linolenic acid (C18:3n-3, LNA) delivered on serum albumin are antiarrhythmic in this dog preparation [4], although the number of animals tested are too few to determine relative efficacy. We purposely infused the n-3 fatty acids rather than fed the dogs fish oil to be certain exactly what ingredient of the fish oil prevented the fatal VF. In dietary studies invariably several things must change, which may confound the study,

but when the free fatty acids were infused intravenously just prior to producing the ischemia and the fatal VF is prevented, then we think we can feel confident that the effect results from what has just been infused.

After this confirmation of the earlier report that polyunsaturated fatty acids, particularly of the n-3 class, prevent ischemia-induced malignant ventricular rhythms, we sought to learn the mechanism of their antiarrhythmic action. Together with Dr Jing X. Kang we adopted the cultured neonatal rat cardiac myocyte preparation for our studies because we could observe and record the contractile function of the isolated myocytes and the effects on their function of adding agents of interest to the medium bathing the myocytes. The beauty of this preparation is that the enzymatically separated neonatal myocytes can be cultured directly on microscope cover slips to which they adhere. By the second day in culture clumps of varying numbers of myocytes can be seen and each syncytial cluster is beating spontaneously, rhythmically and synchronously. With an inverted microscope, video camera with monitor, an edge monitor and tracing recorder, we could observe and record the rate and amplitude of spontaneous contractions [5].

We found that a variety of agents which can cause fatal arrhythmias in humans will cause an acceleration of the beating rate of the myocytes and then produce, asynchronous contractions mimicking fibrillation in the whole heart. Elevated perfusate [Ca^{2+}], cardiac glycoside ouabain [5], catacholamines [6], thromboxane [7], lysophosphatidyl choline or acylcarnitine and even the calcium ionophore A23187 [8] all caused the cultured myocytes to fibrillate. However, if low micromolar concentrations of the fish oil fatty acids eicosapentaenoic acid (C20:5n-3, EPA) or docosahexaenoic acid (C:22:6n-3, DHA) were first added to the bathing medium of the myocyte, their beating rate would slow down. Then when the arrhythmogenic agent was added to the sujperfusate, no arrhythmias occurred. If the arrhythmic agent was added first and an arrhythmia was induced, then addition of the EPA or DHA to the perfusate within a few minutes stopped the arrhythmia in the continued presence of the cardiac toxin. Finally, addition of delipidated serum albumin, which has high affinity binding sites for fatty acids will extract the free fatty acids from the cells and the fibrillation resumes [5].

These simple observations taught us two important things [5]. First, it showed that the antiarrhythmic action of the n-3 PUFAs required the fatty acids simply to partition (dissolve) into the hospitable lipophilic phospholipid acyl chains of the plasma membrane without covalent bonding to any constituent of the cell. Otherwise, the PUFAs could not be extracted from the myocytes by the serum albumin. We had expected the

PUFAs to be rapidly incorporated into membrane phospholipids, but we have shown that once they are so incorporated they are no longer antiarrhythmic [9]. Second, we found that only the free fatty acid is promptly antiarrhythmic. Administering esterified PUFAs, such as their ethyl esters or triglycerides, have no acute antiarrhythmic actions [5].

By this time we realized that the n-3 fatty acids must be affecting the basic automaticity/excitability of cardiac myocytes. Therefore, with Dr. Yong-Fu Xiao we tested their effects on the electrophysiology of the cardiac myocytes. Important effects were found [10]. First, in the presence of these fatty acids (5 to 10 µM) it required a stronger electrical depolarizing stimulus of 40 to 50% simply to elicit an action potential. This was because the presence of these PUFAs caused a slight hyperpolarization of the resting or diastolic membrane potential, while at the same time moving the potential for the gating of the voltage-dependent Na^+ current to more positive values. Second, these fatty acids markedly prolonged the refractory period of the myocytes without any prolongation of duration of the action potential. In fact the action potential duration was slightly but significantly reduced. These electrophysiologic effects are important for the antiarhythmic effects of the PUFAs.

Since all electrical activity of excitable tissues results from the flow of charged ions through specific protein ion channels embedded in the plasma membranes of cells, we turned to examine the effects of the PUFAs on specific membrane ion currents. Dr. Xiao began his study with the effects of the fatty acids on the voltage-dependent Na^+ current, I_{Na}. The PUFAs inhibited the I_{Na} in a concentration dependent manner, with a 50% inhibitory concentration (IC_{50}) of 4.8 µM in neonatal rat cardiomyocytes [11], but only 0.51±0.06 µM in a human embryonic kidney cell line (HEK293t), transiently expressing human myocardial sodium α-subunits ($hH1_\alpha$ [12]. Inhibition occurred within seconds of application of the PUFAs to the myocytes and was reversed as rapidly by the addition of delipidated bovine serum albumin to the perfusate.. It was voltage-dependent, but not use-dependent, consistent with the lipophilic nature of the PUFAs [13]. In both preparations - I_{Na} in the rat cardiomyocyte and $I_{Na\alpha}$ in the human myocardial α-subunit ($hH1_\alpha$) transiently expressed in HEK293t cells - the PUFAs caused a large voltage-dependent shift of the steady state inactivation potential to more hyperpolarized values; the shift at $V_{½}$ = -19 mV with 10 µM EPA in the neonatal rat cardiomyocyte and -27.8 mV with 5 µM EPA in the $hH1_\alpha$. There was no effect of the PUFAs on the activation of the Na^+ channels, only on the inactivated channel. The PUFAs prolonged the inactivated state of the $hH1_\alpha$ channels by speeding the transition from the active to the inactivated state and retarding the slow inactivation phase of the channel [12]. In more

recent studies [14] the β1 subunit has been transiently coexpressed with the α-subunit in HEK293t cells to produce the $hH1_{\alpha\beta}$ and this shifted the steady state inactivation potential to the right (to more depolarized potentials) returning the electrophyiology of the $hH1_{\alpha}$ channels back almost to identity with what we had observed for the neonatal rat cardiomyocytes. EPA was found to have no effect on the activation but only on the inactivation of the Na^+ currents: $I_{Na\alpha\beta}$, $I_{Na\alpha}$ and $I_{Na,rat}$. Consistent with the effects of these fatty acids solely on the inactivated state of the Na^+ channel is the finding that the binding or interaction of these fatty acids to the inactivated state of the Na^+ channels displayed a 265-fold higher affinity for 5 μM EPA than channels in the closed resting, but activatable, state of $hH1_{\alpha\beta}$. [14].

These effects of the n-3 PUFAs (and DHA and LNA do the same as EPA) we think are pertinent to the antiarrhythmic actions of these fatty acids. Our current hypothesis [14] is that this voltage-dependent shift of the steady state inactivation potential to more negative, hyperpolarizing voltages is important to the demonstrated antiarrythmic action of the PUFAs in ischemia-induced fatal arrhythmias. With a coronary thrombosis there occurs a gradient of depolarizations of cardiomyocytes within the ischemic tissue. Cells in the central core of the ischemic tissue quickly depolarize and die due to lack of oxygen and metabolic substrates. Depolarization results from the dysfunctional state of Na, K-ATPase and the rise of interstitial K^+ concentrations in the ischemic tissue. But at the periphery of the ischemic zone myocytes may be only partially depolarized. They become hyperexcitable since their resting membrane potentials become more positive, approaching the threshold for the gating of the fast Na^+ channel. Thus, any further small depolarizing stimulus (e.g., current of injury) may elicit an action potential, which, if it occurs out of phase with the electrical cycle of the heart, may initiate an arrhythmia. In the presence of the n-3 PUFAs, however, a voltage-dependent shift of the steady-state inactivation potential to more hyperpolarized resting potentials occurs. The consequence of this voltage-dependent, hyperpolarizing shift is that the negative potential necessary to return these Na^+ channels from an inactive state to a closed resting, but activatable state, requires a physiologically unobtainable hyperpolarized resting membrane potential. Also these partially depolarized cardiomyocytes have Na^+ channels which within milliseconds can slip into "resting inactivation" from the closed resting state without eliciting an action potential [12]. The result of these two effects of the n-3 PUFAs is that these partially depolarized

myocytes are quickly eliminated from functioning, and their potential arrhythmic mischief is aborted. By contrast, myocytes in the non-ischemic myocardium, with normal resting membrane potential, will not be so drastically affected by this voltage-dependent action of the PUFAs and continue to function normally [14].

Disturbed regulation of cytosolic free calcium concentrations is another cause of malignant arrhythmias occurring in ischemia or resulting from a variety of cardiac toxins. Elevations of cytosolic calcium concentrations can result in tachyarrhythmias. The arrhythmias induced by some cardiac toxins mentioned (elevated extracellular Ca^{2+}, ouabain, catecholamines, etc) are examples of arrhythmias induced by excessive cytosolic Ca^{2+} fluctuations. Such excessive cytosolic free Ca^{2+} fluctuations can induce delayed after-potentials *in vivo*, which may trigger fatal arrhythmias if the after-potentials become sufficient to initiate sodium currents and an action potential occurs at a vulnerable moment in the electrical cycle of the heart. Because both $I_{Ca,L}$ and sarcoplasmic reticulum release of Ca^{2+} underlie many cardiac arrhythmias, together with Drs. A.M. Gomez and W.J. Lederer, the effects of the PUFAs on $I_{Ca,L}$ and Ca^{2+} sparks were examined [15]. Whole-cell voltage clamp techniques and confocal Ca^{2+} imaging were used to determine the effects of PUFAs on the voltage-gated L-type Ca^{2+} current ($I_{Ca,L}$), elementary sarcoplasmic reticulum Ca^{2+}-release events (Ca^{2+}-sparks), and $[Ca^{2+}]_i$ transients in isolated adult rat ventricular myocytes. Extracellular application of eicosapentaenoic acid and the other antiarrhythmic polyunsaturated fatty acids, but not saturated or monounsaturated fatty acids produced a prompt and reversible concentration-dependent inhibition of $I_{Ca,L}$. The concentration of EPA to produce 50% inhibition (IC_{50}) of $I_{Ca,L}$ was 0.8 μM in neonatal rat heart cells and 2.1 μM in adult rat ventricular myocytes. The suppression of the $I_{Ca,L}$ by the PUFAs was voltage and time dependent but not use dependent

When heart cells become "overloaded" with Ca^{2+}, they become arrhythmogenic and produce arrhythmogenic I_{TI} currents and waves of elevated $[Ca^{2+}]_i$ that propagate within the heart cell Thus it seems our finding that the n-3 PUFAs are potent inhibitors of $I_{Ca,L}$ and that this prevents the cytosolic Ca^{2+} overload [8] appears to be the major mechanism by which this cause of triggered arrhythmias evoked by ischemia or cardiac toxins are prevented by the PUFAs.

Both repolarizing potassium currents, the initial fast outward current, I_{to}, and the slow, delayed rectifier current, I_{ks}, were also inhibited but less potently with IC_{50} of 7.5 and 20 μM of DHA, respectively. The inward rectifier potassium current, I_{k1}, by contrast, was not affected by the PUFAs [Xiao, Morgan, AL, unpublished results]. The net effect of inhibiting the two main repolarizing K^+ currents should result in prolongation of the action potential duration, but the action potential was shortened slightly, but significantly by the PUFAs. This action together with the higher concentrations of PUFAs required to inhibit these currents make us think that the effects of PUFAs on K^+ currents are not playing a significant role in their antiarrhythmic effects.

To demonstrate that these electricophysiologic actions of the fatty acids result in stabilizing the cardiac myocytes electrically a simple experiment was designed [8]. While making a continuous tracing of the regular spontaneous beating rate of the cultured neonatal rat cardiomyocytes, the myocytes could be stimulated with an external voltage source at 15 volts via two platinum electrodes immersed in the perfusion fluid at the two ends of the perfuion chamber. It was easy with this set up to double the beating rate of the myocytes. When the external electrical stimulator was turned off, the contractions of the myocytes returned to their control beating rate. Adding the n-3 PUFA to the perfusate slowed the beating rate of the myocyte, but when the external electrical stimulus was again turned on at 15 volts, the myocytes paid no attention to the stimulii. At 20 volts the myocytes still did not respond. At 25 volts they did respond, but only to every other electrical stimulus. Now, adding delipidated bovine serum albumin to the perfusing fluid and extracting the free fatty acids from the myocytes, the beating rate again returned to its control rate. When the external electrical stimulator was again turned on at 15 volts, the myocytes doubled their beating rate, just as they had prior to exposure to the n-3 PUFAs. This experiment was done on isolated cardiac myocytes in the absence of humoral or neural regulation. When one considers that this is a direct action of the free n-3 fatty acids on every individual myocyte in the heart, one can appreciate what a potentially effective antiarrhythmic agent they are. Furthermore, their electrical stabilizing effect on each cardiac myocyte is independent of the cause of arrhythmias, whether the cause is ischemia, increased cytosolic free calcium fluctuations, or other, the n-3 PUFAs seem capable of exerting their antiarrhythmic effects.

It is this last observation that gives me some hope, that on a prophylactic basis administering the n-3 polyunsaturated fatty acids in fish oils on a daily regimen might prevent sudden cardiac death in patients with the Brugada syndrome. I hasten to state that I have no experience with subjects having the Brugada syndrome. But from an anecdotal consulting relation to the care of a very few patients with potentially fatal ventricular arrhythmias arising from genetic impairment of cardiac ion channel function (i.e., long QT syndromes), it seems not unreasonable to recommend a clinical trial of such prophylaxis in patients with the Brugada syndrome.

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References

1. McLennan PL, Abeywardena MY, Charnock JS: Dietary fish oil prevents ventricular fibrillation following coronary artery occlusion and reperfusion. Am Heart J 1998; 116:709-17.

2. Schwartz PJ, Billman GE, Stone HL Autonomic mechanisms in ventricular fibrillation induced by myocardial ischemia during exercise in dogs with healed myocardial infarction: an experimental preparation for sudden cardiac death. Circulation 1984; 69: 790-800.

3. Billman GE, Kang JX, Leaf A. Prevention of ischemia-induced cardiac sudden death by n-3 polyunsaturated fatty acids in dogs. Lipids 1997; 32: 1161-1168.

4. Billman GE, Kang JX, Leaf A: Prevention of ischemia-induced cardiac sudden death by pure n-3 polyunsaturated fatty acids. Circ 1999: 99, 2452-2457.

5. Kang JX, Leaf A: Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes. Proc Natl Acad Sci USA 1994; 91:9886-9890.

6. Kang JX, Leaf A: Prevention and termination effect of the β-adrenergic agonist-induced arrhythmias by free polyunsaturated fatty acids in neonatal rat cardiac myocytes. Biochem Biophys Res Comm 1995; 208:629-636.

7. Li Y, Kang J.X., Leaf A: Differential effects of various eicosanoids on the production or prevention of arrhythmias in cultured neonatal rat cardiac myocytes. Prostaglandins 1997; 54: 511-530.

8. Kang JX, Leaf A: Prevention and termination of arrhythmias induced by lysophosphatidyl choline and acylcarnitine in neonatal rat cardiac myocytes by free omega-3 polyunsaturated fatty acids. Eur J Pharmacol 1996; 297: 97-106.

9. Weylandt K H, Kang JX, Leaf A: Polyunsaturated fatty acids exert their antiarrhythmic actions as free fatty acids rather than in phospholipids. Lipids 1996; 31: 977-982.

10. Kang JX, Xiao YF, Leaf A: Free long-chain polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. Proc Natl Acad Sci USA 1995; 92: 3997-4001.

11. Xiao Y-F, Kang JX, Morgan JP, Leaf A: Blocking effects polyunsaturated fatty acids on Na^+ channels of neonatal rat ventricular myocytes. Proc Natl Acad Sci USA 1995; 92: 1000-11004.

12. Xiao Y-F, Wright SN, Wang GK, Morgan JP, Leaf A: N-3 fatty acids suppress voltage-gated Na^+ currents in HEK293t cells transfected with the α-subunit of the human cardiac Na^+ channel. Proc Natl Acad Sci USA 1998; 95: 2680-2685.

13. Hille B: Local anesthetics: hydrophilic and hydrophobic pathyways for the drug-receptor reaction. J Gen Physiol 1977; 69: 497-515.

14. Xiao, Y-F, Wright SN, Wang GK, Morgan JP, Leaf A: Coexpression with the β_1 - subunit modifies the kinetics and fatty-acid block of $hH1_\alpha$ Na^+ channels. Am J Physiol Heart Circ Physiol 2000; 279: H35-H46.

15. Xiao Y-F, Gomez AM, Morgan JP, Lederer WJ, Leaf A: Suppression of voltage-gated L-type Ca^{2+} currents by polyunsaturated fatty acids in adult and neonatal rat cardiac myocytes. Proc Natl.Acad Sci USA 1997; 94: 4182-4187.