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Review

Antiarrhythmic fatty acids and antioxidants in animal and cell studies

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From the animal and cellular studies that will be discussed in this review, it is apparent that dietary fatty acids and antioxidants play an important role in influencing the development of ventricular tachycardia and potentially lethal ventricular fibrillation. It is this latter disturbance to the rhythmic beating of the heart that is responsible for much of the mortality from coronary heart disease. It is now recognized that diets high in certain polyunsaturated fatty acids (PUFAs) and diets containing antioxidants can afford considerable protection to the heart with regard to the generation of disorders of contractile rhythmicity. The mechanism by which such dietary components confer their cardioprotective effects are now being intensively investigated, particularly with respect to their possible effects on the molecular mechanisms underlying the excitation-contraction coupling process of the myocardial cell. This overview will cover recent studies that have focused on the antiarrhythmic role of PUFAs, particularly those of the n-3 (or omega 3) class with emphasis on experiments performed using laboratory animals, isolated heart preparations, and isolated heart cells (cardiomyocytes). The role of free radicals (reactive oxygen species) and antioxidants in disorders of cardiac rhythm also will be addressed within the perspective of reperfusion injury to the myocardium following ischemia. Emphasis will be placed on the cardioprotective role of nutritional factors and components and the possible cellular mechanisms by which such components may act. (J. Nutr. Biochem. 10:252–267, 1999) *© Elsevier Science Inc. 1999. All rights reserved.*

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Introduction

Coronary heart disease (CHD) remains one of the leading causes of mortality in many industrialized countries.[1] The major clinical manifestations of CHD include myocardial infarction, cardiac arrhythmias, and sudden cardiac death. Cardiac arrhythmia can occur during the early phase of ischemia and, in certain situations, following the restoration of normal blood flow (reperfusion) to the ischemic region of the myocardium. Individual heart cells are electrically coupled both to each other and to the conducting pathways by gap junctions, which allow electrical conductance to pass from one cell to the next.[2] During ischemia, the electrical properties of the heart are changed, resulting in arrhythmias such as ventricular tachycardia and ventricular fibrillation (VF), which can lead to sudden cardiac death. Although arrhythmias can be of many types and vary in their etiology, the inability of individual cardiomyocytes to function properly is fundamental to the generation of arrhythmias.

Useful strategies for reducing the incidence of CHD mortality in the population can be directed either at disease prevention or at improving the treatment for patients with known symptoms. Many of the risk factors associated with CHD are nutrition-related and therefore modifiable. Risk factors such as the intake of saturated fat, high blood pressure, age, smoking, obesity, and diabetes are known to be related to the development of CHD.[3] More recent evidence suggests that consumption of certain types of polyunsaturated fatty acids (PUFAs) in preference to saturated fats may reduce both CHD incidence and mortality.[4,5] Furthermore, there is increasing evidence that certain micronutrients that possess antioxidant activities such as

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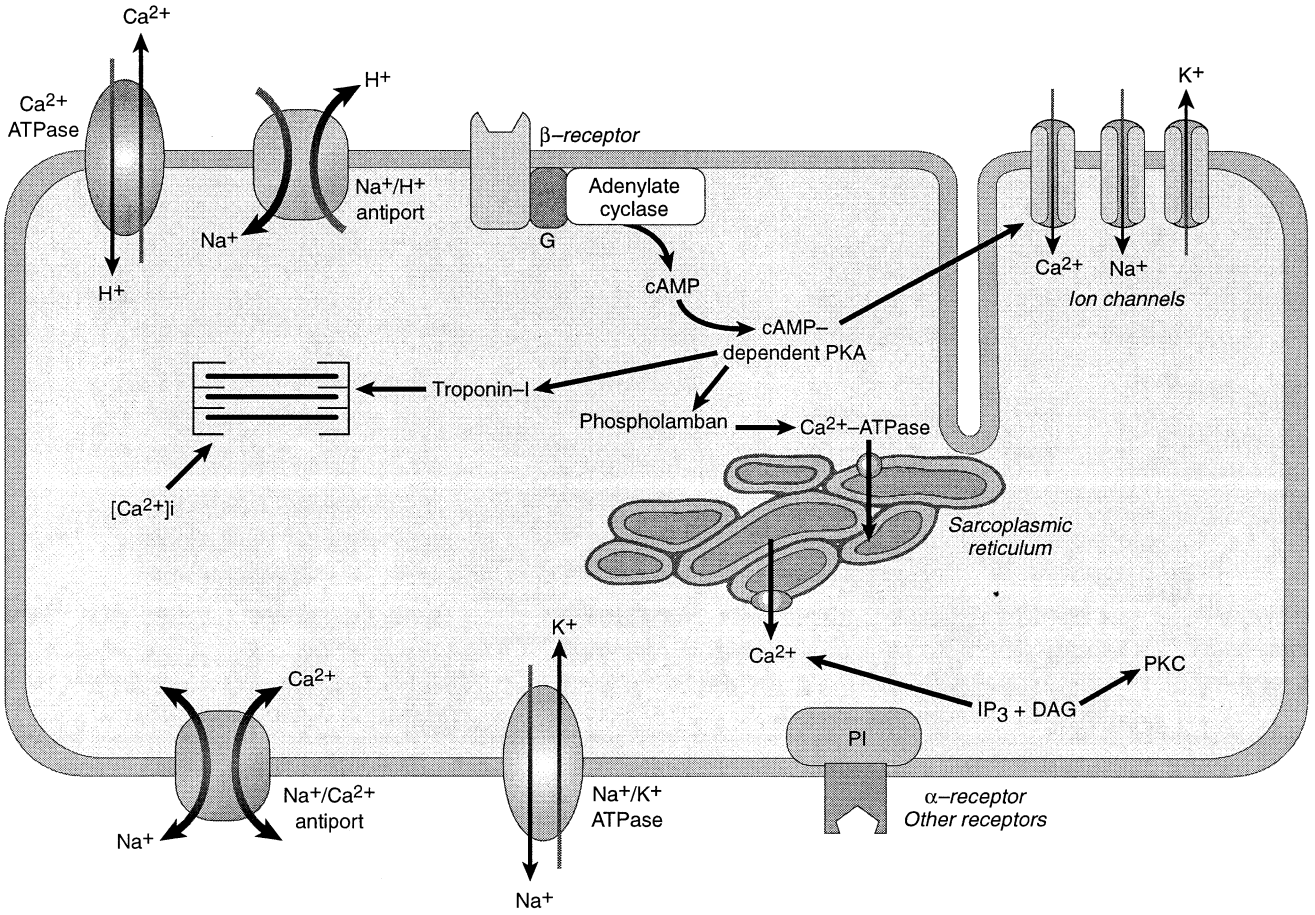

Figure 1 Some of the major ion transporters affected during myocardial ischemia resulting in accumulation of intracellular Ca^{2+} and subsequent contracture. G, G-protein; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PI, phosphoinositide.

phytochemicals including flavonoids may also be protective against CHD.[6–11] Various dietary components including fats, oxidized fats, antioxidants, and phytochemicals, which affect the type and amount of circulating lipids and/or the free radical flux and status within the body, also have the potential to indirectly influence the generation of arrhythmias by their effects on blood vessel function.

Cardiac arrhythmias

Ischemic arrhythmias

Myocardial ischemia exists when the reduction of coronary flow is so severe that the supply of oxygen (and other substrates) to the myocardium is inadequate for the oxygen demands of the tissue.[12] Lack of oxygen supply to the mitochondria results in a rapid decrease in adenosine triphosphate (ATP) synthesis, which impacts on many processes underlying the normal excitation-contraction coupling cycle of the myocardium. A number of sequelae develop as a result of ischemia. Internal Na^+ increases rapidly at the time of ischemia. The Na^+/H^+ exchanger operates to expel intracellular H^+ (which can impair contractility) in exchange for extracellular Na^+.[13,14] This accumulation of intracellular Na^+ leads to further increases in intracellular Ca^{2+} via the Na^+/Ca^{2+} antiporter (*Figure 1*). Following ischemia, potassium is released from cardiomyocytes and there is an increase in intracellular lactate and inorganic phosphate levels and a decrease in intracellular pH. Changes in the activity of K^+ channels also can occur as a result of the decline in cellular ATP. The increased Ca^{2+} also will affect the action of phospholipases, enhancing the liberation of free fatty acids from membrane phospholipids. In addition, the accumulation of lipid metabolites is increased and may have adverse lytic effects on the cell. Delayed afterdepolarizations and triggered automaticity have been also described in the genesis of ischemic arrhythmias.[15,16] Characteristic changes in the electrocardiogram (ECG) pattern include shortening of the action potential duration and ST-segment deviations during ischemia.[12]

Reperfusion arrhythmias/injury

Prolonged ischemia can cause serious damage to the myocardium[17]; however, restoration of flow may not necessarily restore normal contractile function. Instead, contractile function and cardiac viability may become seriously compromised during the very early stages of reflow. Reperfusion injury is observed under a number of clinical circum-

stances[18–23] and encompasses a spectrum of events including reperfusion arrhythmias, myocardial stunning, microvascular damage, and accelerated death of the more severely damaged cells despite reperfusion of the tissue.[12] Reperfusion injury has the potential to occur under four clinical conditions: following relief of coronary artery spasm,[24,25] during aorto-coronary bypass surgery,[26] during balloon angioplasty of the coronary arteries,[27] or following thrombolytic therapy.[28]

Two theories have been proposed to explain the underlying basis of reperfusion injury. The calcium hypothesis proposes that ischemia induces a defect in the ability of the cell to regulate calcium such that upon reperfusion, the cell accumulates toxic levels of calcium. The second theory involves a role for free radicals and reactive oxygen species (ROS). This is based on the premise that partially reduced forms of molecular oxygen are produced at the time of reperfusion.[29] It has been suggested that free radicals per se may be inducing the membrane defects that promote calcium entry, thus unifying both hypotheses.[17] In contrast, Obata et al.[30] reported that calcium overload induced by ouabain resulted in the generation of hydroxyl free radicals. Granger et al.[31] proposed that during ischemia the breakdown of high-energy phosphate compounds results in the accumulation of the purine metabolites hypoxanthine (HX) and xanthine. As the energy charge drops via ATP depletion, the cell no longer maintains normal ion gradients across various membranes and within intracellular compartments, resulting in a redistribution of calcium ions. The elevated intracellular calcium is believed to activate a protease capable of converting xanthine dehydrogenase (XD) to xanthine oxidase (XO).[31] During reperfusion, the sudden re-introduction of oxygen permits the XO catalyzed oxidation of HX with the simultaneous reduction of oxygen to the superoxide ($O_2 \cdot^-$) free radical and hydrogen peroxide (H_2O_2). $O_2 \cdot^-$ and H_2O_2 can then secondarily generate the highly reactive hydroxyl radical ($\cdot OH$) via the Haber-Weiss reaction (*Figure 2*). This overproduction of oxygen-derived free radicals may then overload the cell's natural scavenging mechanisms, causing cellular damage.[32] This may compromise membrane ion pump activity and promote local electrophysiologic derangement(s) sufficient to trigger ventricular arrhythmias.[21] Indeed free radical production has been reported in humans following coronary angioplasty.[33] In a study of CHD patients, those with unstable angina pectoris had higher levels of circulating lipid hydroperoxides, thiobarbituric acid reactive substances (TBARS), and conjugated dienes, and lower α-tocopherol content per low density lipoprotein particles in their plasma compared with subjects with stable angina pectoris and controls.[34,35]

Dietary lipids and the synthesis of polyunsaturated fatty acids

Fatty acids are typically classified into saturated, monounsaturated and polyunsaturated fatty acids. Animal fats are the major dietary source of saturated fatty acids which include palmitic and stearic acids. Oleic acid is a monounsaturated fatty acid found in most of the common edible oils such as olive oil, sunflower oil, safflower oil, and canola oil. Naturally occurring PUFAs typically contain an even number of carbon atoms (between 18 and 24) and are incorporated into the phospholipids within the membranes of cells where they are esterified onto hydroxyl groups in the phospho-glycerol backbone.[36] Mammals are able to synthesize all fatty acids de novo except the "essential" parent fatty acids linoleic acid (LA; 18:2, n-6) and α-linolenic acid (αLNA; 18:3, n-3). These fatty acids are categorized as essential because humans lack the enzymes necessary to insert double bonds between the terminal methyl carbon and the ninth carbon atom. Therefore, LA and αLNA must be obtained from the diet. LA is found in plant seed oils such as sunflower, safflower, olive, and cottonseed oils, and αLNA is found in canola, soybean, and linseed oils.

The synthesis of PUFAs proceeds via a series of reactions involving classes of enzymes that insert carbon atoms (normally two) into the fatty acid chain (elongases), and enzymes that insert double bonds at specific regions of the fatty acid chain (desaturases), leading to increased unsaturation. The original concept of Brenner[37] that a delta-4 desaturase may be involved in this process has now been largely superseded by the recent work of Sprecher and his group, who have reported that during long-chain PUFA synthesis, peroxisomes and β-oxidation participate in the synthesis of the long chain n-3 PUFAs docosahexaenoic acid (DHA; 22:6, n-3) and eicosapentaenoic acid (EPA; 20:5, n-3) by way of C24 PUFA intermediates.[38] *Figure 3* summarizes the pathways of metabolism for the n-6 and n-3 PUFAs.

Arachidonic acid (AA; 20:4, n-6) and EPA also act as precursors for the eicosanoids (bioactive metabolites of AA and EPA). The generation of these latter components in cardiac tissue, particularly with regard to the prostaglandin to thromboxane ratio, have been implicated in arrhythmogenesis[39] and will be discussed later. The n-3 fatty acid family includes the essential fatty acid αLNA and the very long chain PUFAs EPA and DHA, which are found in the marine phytoplankton consumed by fish and in fish per se. In the presence of dietary αLNA, humans are only able to synthesize EPA and DHA de novo at a relatively slow rate by elongation and desaturation of αLNA (*Figure 3*). The consumption of fish or fish products substantially increases the amounts of EPA and DHA available for membrane incorporation and cellular processes.[40] Lack of a dietary supply of essential fatty acids leads to elevation of Mead acid (20:3, n-9), which is a marker for essential fatty acid deficiency, possibly as a consequence of a compensatory mechanism to offset the reduced levels of long chain PUFAs.

Dietary PUFAs in human studies

Scientific interest in the health benefits of the n-3 PUFAs was generated by the epidemiologic studies of Bang and Dyerberg and their colleagues[41–43] who reported that Greenland Eskimos, whose dietary intake from marine sources averaged 500 g/day, had extended bleeding times.

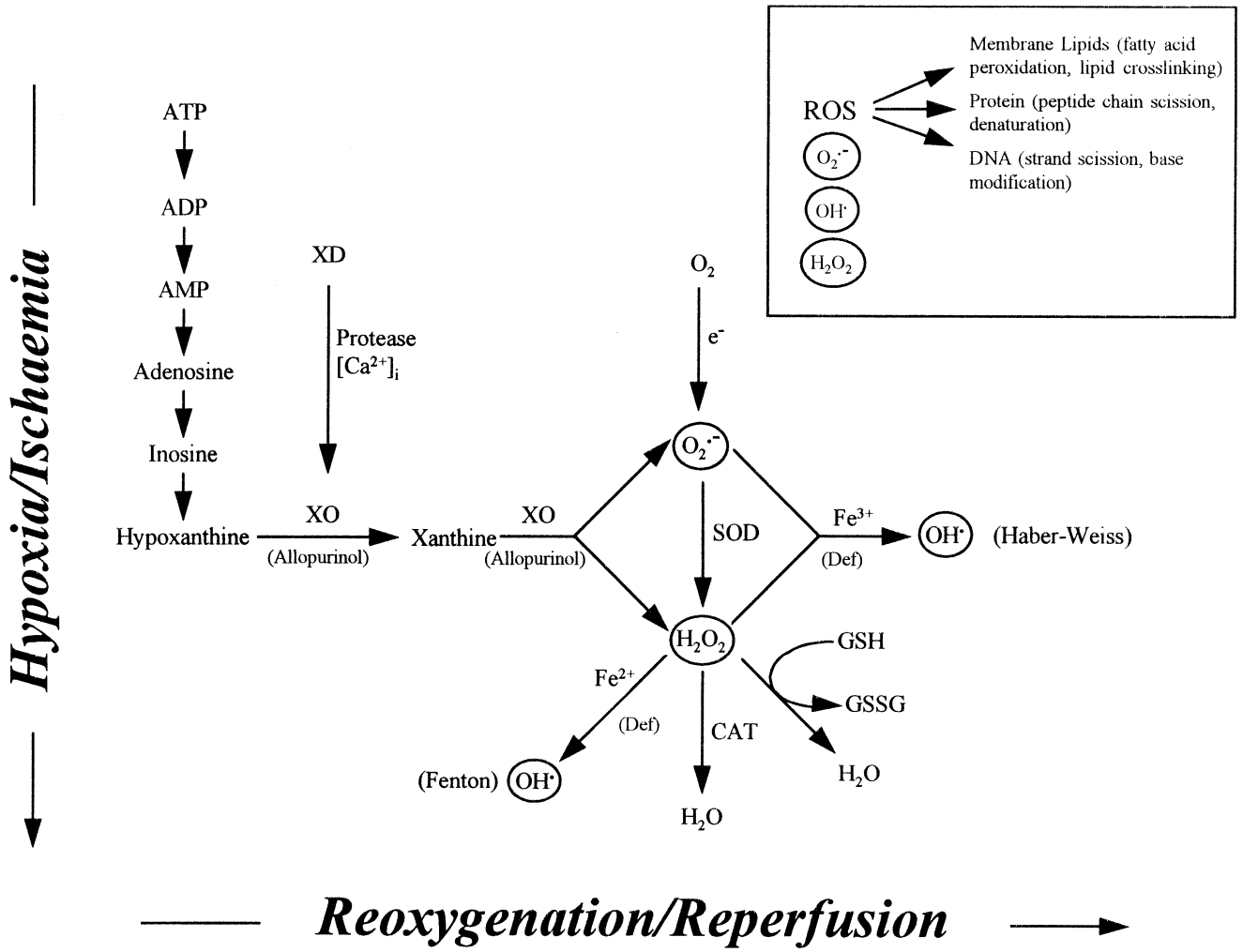

Figure 2 Cellular mechanisms for reactive oxygen species generation during ischemia/reperfusion. During ischemia, adenosine triphosphate (ATP) is degraded to hypoxanthine whereas xanthine dehydrogenase (XD) is converted to xanthine oxidase (XO). At reperfusion hypoxanthine (HX) and XO react with O_2 generating superoxide radical ($O_2 \cdot^-$), and hydrogen peroxide (H_2O_2). $O_2 \cdot^-$ also can be generated by leakage of electrons to O_2 from various components of the cellular electron transport chains. $O_2 \cdot^-$ is dismutated by superoxide dismutase (SOD) forming H_2O_2. H_2O_2 can generate ·OH via the Fenton or Haber-Weiss reaction or can be converted to H_2O by action of catalase (CAT) or glutathione peroxidase. ·OH, hydroxyl radical; Def, deferroxamine; GSH, glutathione; GSSG, reduced glutathione; ROS, reactive oxygen species.

Later studies revealed that this population had significantly lower levels of total and low density lipoprotein cholesterol and a relatively lower incidence of CHD when compared with a Danish study population.[44] The potential benefits of consuming fish and fish oil have been described in several population and clinical studies with particular reference to their potential role in preventing cardiac arrhythmias and sudden cardiac death. In a 20-year follow-up study, mortality from CHD was found to be inversely related to fish consumption.[45] In the Diet and Reinfarction Trial (DART study) the incidence of mortality due to ischemic heart disease was significantly lower in a group of postmyocardial infarction patients advised to include fish in their diet.[46] Similarly, the beneficial cardioprotective effects of n-3 fatty acids have been reported in cardiac patients.[4,47,48] Recently, Singh et al.[5] concluded that fish oil rapidly protects against reperfusion injury in patients suffering acute myocardial infarction.

Dietary antioxidants

Numerous reports confirm the role of free radicals in ischemic/reperfusion damage.[29,32,49–52] Antioxidants are necessary to prevent the formation of free radicals and inhibit some of the deleterious actions of reactive oxygen and nitrogen species that damage DNA, lipids, and proteins.[53] Cellular mechanisms exist to counteract the effects of free radicals and these comprise several antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (*Figure 2*). However, during extreme oxidative stress, the endogenous antioxidant system may be insufficient to scavenge all free radicals produced and consequently, diet-derived antioxidants are likely to play an important defensive role. α-Tocopherol, the major constituent of the fat-soluble vitamin E, is the most important chain-breaking antioxidant within membranes and lipoproteins.[53] This antioxidant inhibits lipid

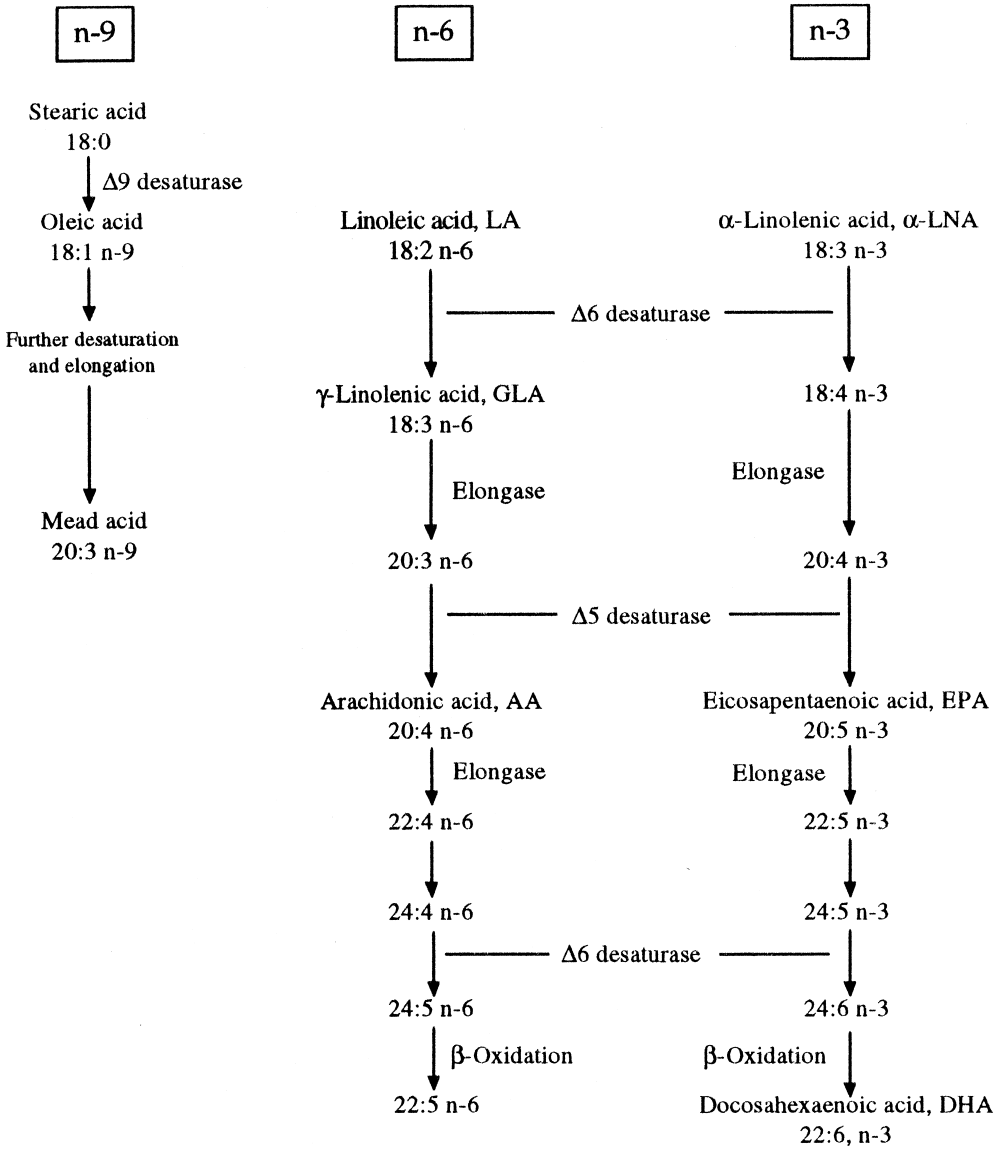

Figure 3 Schematic representation of the n-6 and n-3 series metabolic pathways.

peroxidation by scavenging peroxyl radicals generated from PUFAs in membrane phospholipids.[54] The resulting α-tocopherol radical (although not completely unreactive) is much less reactive than the peroxyl radical and therefore acts as a chain-breaking antioxidant.[53] Vitamin C is a potent antioxidant in extracellular fluids and efficiently scavenges $O_2 \cdot^-$, H_2O_2, $\cdot OH$, hypochlorite ions, peroxyl radicals, and singlet oxygen.[54] It also may facilitate the regeneration of α-tocopherol from the radical form.[53] β-Carotene, a precursor for vitamin A, efficiently quenches singlet oxygen, thereby protecting biological systems against singlet-oxygen mediated damage.[54] Recently, much interest has been centered on the flavonoids, a group of naturally occurring low molecular weight benzo-γ-pyrone derivatives that are ubiquitous in the photosynthesizing cells of plants. Flavonoids have been reported to possess antioxidant, anti-inflammatory, antiallergic, and antihemorrhagic properties.[55] Their antioxidant action has been attributed to both their free radical scavenging capacity[56] and iron-chelating ability.[57] It has been suggested that flavonoids in red wine could explain the so-called "French paradox" relating to dietary fat and cardiovascular disease and red wine consumption.[58]

Antioxidant studies in humans

Increased production of ROS is a feature of many human diseases including cardiovascular disease. Dietary antioxidants may be important in protecting against diseases associated with free radical damage to DNA, lipids, and proteins.[59] Thus, numerous epidemiologic studies have demonstrated an association between dietary and supplemental intake of antioxidant vitamins and decreased mortality and morbidity from CHD.[60,61] Vitamin C, carotenoids, and vitamin E, which are the three main dietary

sources of antioxidants, can each influence lipid peroxidation and may decrease atherosclerosis, thereby lowering the risk of CHD.[62] The evidence of a cardiovascular benefit of antioxidants is strongest for vitamin E.[7,8,63] Vitamins E and C supplemented together are also associated with a lower risk of total mortality in the elderly.[6] Results from recent clinical trials of β-carotene and vitamin E supplementation report no cardiovascular benefit[64–67]; however, some studies have found an inverse association between carotenoid intake or plasma levels and risk of CHD.[9,10] A cardioprotective role for the flavonoids was demonstrated in the Netherlands where an intake of 26 mg flavonoids per day was found to be inversely associated with mortality from CHD.[11]

Animal models of ischemic and reperfusion arrhythmias

Different animal models of cardiac arrhythmia have been used to study the relationship between dietary lipids, cardiac membrane lipid composition, myocardial function, and the biochemical mechanisms underlying antiarrhythmic effects in relation to nutritional components. A number of studies have investigated the protection afforded by various nutritionally-derived or related agents on arrhythmias induced in the isolated or ligated heart model using both dietary and acute addition of these putative antiarrhythmic compounds. Antiarrhythmic properties of PUFAs were first reported in animal models of arrhythmia by Murnaghan.[68] Subsequently, McLennan et al.[69] reported that diets high in saturated fats were associated with a relatively higher incidence of VF in rats when myocardial ischemia was induced by coronary artery ligation in situ. Dietary sunflower oil (LA rich) reduced the incidence of ischemia-induced ventricular arrhythmias by approximately 30% compared with animals maintained on a diet supplemented with saturated fat, whereas tuna fish oil high in n-3 PUFAs completely prevented both ischemia and reperfusion-induced arrhythmias.[69–72] These findings were confirmed in marmosets fed diets containing a mixture of sheep fat (saturated fat) and sunflower seed oil compared with animals fed sheep fat combined with fish oil for 16 weeks.[72] The VF threshold under programmed electrical stimulation was elevated significantly in the fish oil group compared with the sunflower seed oil group and this was associated with increased levels of n-3 fatty acids incorporated into the myocardial membrane phospholipids.

McLennan and Dallimore[73] reported that following 15 minutes of ischemia induced by in vivo coronary artery ligation, rats fed an olive oil supplemented diet for 12 weeks exhibited a higher incidence of VF than rats fed a canola oil enriched diet where no VF events and a lower arrhythmia score were recorded. If the duration of ischemia was shortened to 5 minutes, the canola oil fed animals again exhibited a lower arrhythmia score, a tendency to fewer VF events and no fatal VF in comparison with olive oil fed animals. Although protection afforded by the n-3 PUFAs may be due to the coincident effect of the reduced saturated fatty acid content, it has been reported that animals fed a fish oil supplemented diet are consistently protected from developing arrhythmias compared with animals fed n-6 PUFA and saturated fatty acid diets, such that their effects do not correlate with the relative amounts of saturated and polyunsaturated fatty acids per se.[69,70,72] Additionally, canola oil, which like olive oil is composed mainly of oleic acid (which is not antiarrhythmic) but also contains approximately 8% αLNA, offers significant antiarrhythmic protection.[73] The antiarrhythmic effect of the canola oil cannot be attributed solely to the presence of the n-3 PUFA αLNA, because soybean oil which contains similar concentrations of αLNA is not antiarrhythmic,[73] but rather to the fact that the LA present in soybean oil competes with αLNA, preventing its conversion to the longer chain n-3 PUFAs, which are potently antiarrhythmic.

Rats fed a fish oil diet for 16 weeks were protected against the development of arrhythmias following ischemia and reperfusion when blood-perfused, electrically-paced working hearts were investigated.[74] This protection by a fish oil diet was associated with an increase in n-3 PUFA incorporation into the myocardial phospholipids. In hearts allowed to spontaneously contract, VF could be induced by programmed stimulation. Rats fed fish oil required a significantly higher stimulation current to induce VF compared with saturated-fat fed rats.[74] Hock et al.[75] reported that the protection afforded against arrhythmias induced in situ by coronary artery ligation and reperfusion by a fish oil diet (4 weeks) was associated with reduced leukocyte infiltration in the left ventricular wall. Leukocytes can release a number of potentially deleterious substances including free radicals which would promote myocardial necrosis. These authors suggested that the fish oil diet may be providing protection by selective incorporation of n-3 PUFAs into leukocyte membrane phospholipids, leading to inhibition of phospholipase activity with a resultant reduction in lipoxygenase metabolite production.[75] However, in isolated hearts following reperfusion, Yang et al.[76] showed a protective effect of fish oil independent of its effects on plasma. Using a perfusate free of plasma and circulating cellular elements such as platelets and leukocytes, reperfusion injury was lower in hearts from animals fed a fish oil diet for 5 days.

More recent studies by McLennan et al.[77] demonstrated that dietary supplementation of purified DHA mimics the actions of fish oils. The antiarrhythmic effects of free n-3 PUFAs have also been observed following slow intravenous infusion (40–60 minutes) of the free n-3 PUFAs in arrhythmia susceptible, conscious dogs.[78] The antiarrhythmic effect of the infused free n-3 PUFAs was associated with a reduction in heart rate, shortening of the action potential duration, and prolongation of the ECG atrial-ventricular conduction time.

Intravenous infusion of the flavonoid quercetin administered 2 minutes prior to reperfusion also prevented reperfusion-induced arrhythmias in vivo in the anesthetized rat.[79] Furthermore, protection was associated with an inhibition of platelet aggregation and thromboxane A_2 formation. Quercetin also has been shown to be protective against reperfusion injury following occlusion and reperfusion of the coronary artery in dogs,[80,81] and also following hypoxia and hyperthermia,[82] due to its antioxidative and inhibitory effects on lipoxygenase activity. Further protection by the flavonoids was demonstrated in situ in the anesthetized rat

where intravenous infusion of quercetin and silybin administered 15 minutes prior to ischemia prevented the decrease in the XD:XO ratio occurring during ischemia/reperfusion in rat kidney.[83] Furthermore, in normal rat kidney, these flavonoids exerted a concentration-dependent inhibition on the activity of XO. Thus, the protective effects of quercetin during ischemia/reperfusion may be attributed to the inhibition of XO activity or alternatively, to the inhibition of XO formation. However, quercetin has multiple effects that may not allow clear conclusions regarding its cardioprotective mechanisms.[84] Another flavonoid, purpurogallin (PPG) administered intravenously 1 minute prior to reperfusion was shown to decrease myocardial damage in rabbits following 1 hour ligation of the anterior coronary artery.[85] Acute addition of PPG also was reported to protect isolated human ventricular, endothelial, and red blood cells against damage from free radicals generated by the free radical generating systems, HX/XO, menadione (generating $O_2 \cdot^-$), and paraquat (generating H_2O_2 and $\cdot OH$). The protection afforded by PPG was more potent than the antioxidants trolox (the hydrophilic region of the α-tocopherol molecule), vitamin C, and mannitol ($\cdot OH$ scavenger).[86] The use of human cells in this study, particularly cell types that are intimately involved in myocardial infarction, offers greater clinical relevance than animal based studies and adds further support to the experimental studies.

van Jaarsveld et al.[87] found that supplementing the drinking water of rats with pycnogenol (proanthocyanidin) increased the amount of α-tocopherol and ascorbic acid detectable in myocardial tissue. However, despite this increase, pycnogenol was not able to reduce the extent of mitochondrial damage and myocardial low molecular weight iron increase upon reperfusion following a protocol of normothermic ischemic cardiac arrest. In contrast, the addition of catechin to the perfusate in the isolated heart system was protective. Catechin also prevented the decrease in the ascorbic acid content of the myocardium induced by ischemia/reperfusion. An inhibitory effect of tea catechins on XO activity has been reported, which may partly explain the antioxidant effects of catechin.[88] Further support for the role of XO in reperfusion arrhythmogenesis comes from the study of Manning et al.[89] in which it was reported that the XO inhibitor allopurinol was effective in preventing reperfusion-induced VF in the rat following transient coronary artery occlusion.

Using ascorbyl free radicals (AFR) detected by electron spin resonance spectroscopy, an increase in the level of AFR following ischemia and reperfusion has been reported in isolated rat hearts.[90] A slow constant release of AFR occurred during low flow ischemia; however, upon reperfusion, there was a sudden and large burst of AFR liberation, which was further enhanced if the duration of ischemia was increased from 20 minutes to 60 minutes. These results strongly support the role of free radicals in reperfusion injury and suggest that free radical production at the time of reperfusion depends on the duration and extent of the preceding ischemia.

Reperfusion after 30 minutes of no-flow ischemia in the right ventricular wall of the guinea-pig was associated with premature action potentials and the development of tachycardias.[91] Pretreatment with SOD, CAT, and mannitol for 20 minutes prior to ischemia reduced the incidence of tachycardias and cell membrane damage. This indicates that a combination of H_2O_2, $O_2 \cdot^-$, and $\cdot OH$ are involved in the myocardial damage following ischemia/reperfusion. Further support for free radical mediated ischemia/reperfusion injury comes from studies indicating protection by SOD and CAT,[92] the vitamin E analogues raxofelast[93] and MDL 74366,[94] the indenoindole compound H290/51,[95] and all-*trans*-retinoic acid.[96]

Arrhythmia studies in isolated cardiomyocytes

Studies using isolated cardiomyocytes to investigate mechanisms by which dietary components exert their antiarrhythmic properties have both advantages and disadvantages compared with whole animal in situ heart studies or isolated heart studies. Dietary studies designed to determine the effects on the heart at the in vivo level may be confounded by neural and humoral influences, variable blood pressure and heart rate, circulating fatty acids, or other extracardiac effects influenced by the dietary lipid intake. Studies at the cellular level enable the investigation of the direct effect of fatty acids on the cardiomyocyte as well as the determination of the possible cellular mechanism(s) involved in cardioprotection by both infused and incorporated fatty acids or antioxidants. Isolated ventricular cardiomyocyte preparations have the potential to be used for arrhythmia studies, particularly with regard to investigating the efficacy of as well as the underlying mechanisms whereby certain dietary components are able to exert their cardioprotective effects.

Many clinically encountered arrhythmias result from the phenomenon of re-entry and this can arise equally in many areas of the heart such as from a bundle of conducting fibers to an area containing working myocardial cells.[97] The phenomenon of re-entry and subsequent generation of re-entrant arrhythmic activity in the form of premature systoles and tachycardia results from the presence of an area of decremental conduction that exhibits slowed impulse conduction together with a unidirectional block. Therefore, normal propagation of the impulse conduction wave through areas of the functional syncytium, be they conducting fibers or muscle cells, can be perturbed in such areas due to damage arising from, for example, heart failure or the imposition of ischemia. Through secondary processes such as summation and inhibition of the conduction wave arising from these conduction blocks, re-entrant pathways are established and the syncytium no longer functions as an effective integrated unit, but through these mechanisms displays many of the types of arrhythmic activity encountered clinically.[97]

Cultured neonatal cardiomyocytes which contract rhythmically and synchronously in a syncytium have been used extensively in the study of the cardioprotective effects of n-3 PUFAs and other compounds.[1,98–103] Neonatal cells display differences in intracellular morphology compared with cells derived from adult animals particularly with regard to the stage of development of the sarcoplasmic reticulum (SR) and the relative contributions of intracellular/extracellular calcium handling to the excitation-contraction cycle. Cardiomyocytes isolated from adult animals are

calcium tolerant and quiescent and remain so over several days. Many cardiomyocytes can be obtained from the heart of an adult animal, which permits numerous experimental manipulations to be performed using only small amounts of experimental compound(s). Furthermore, the actual concentration of such compounds at the cell surface can be determined accurately. Obvious disadvantages are the fact the cells are not under load when contracting, have been subject to the calcium paradox during isolation (raising $[Ca^{2+}]_o$ from low to high concentrations quickly during cell isolation can cause hypercontracture), and consequently, may have sustained damage to many regions including the gap junction area. However, isolated cardiomyocytes behave in a predictable manner with regard to mimicking the behavior of the whole heart to an extremely wide range of pharmacologic agents and changing physiologic conditions. Adult cardiomyocytes display similar contractile properties to the intact tissue.[104,105] Elevated $[Ca^{2+}]_i$ levels have been implicated in the progression of triggered cardiac arrhythmia in a variety of conditions such as those following myocardial ischemia and reperfusion, as well as exposure to catecholamines and digitalis (cardiac glycosides).[97] Isolated cardiomyocytes respond to an increased $[Ca^{2+}]_o$ with the development of extra beats, tachyarrhythmias, chaotic beating activity, afterdepolarisations, and triggered contractile activity,[106–116] an arrhythmia profile similar to that recorded from the isolated or in vivo heart.[117]

To make some extrapolation between isolated cardiomyocytes and the situation in the whole heart, the fact that the imposition of arrhythmic stimuli, which induce cells to beat in a manner out of synchrony with an applied electrical stimulus, indicates that an isolated cardiomyocyte has the potential to develop all of the characteristics that would lead it, in association with neighboring cells (were it within the heart itself), to give rise to a region of decremental conduction in the working myocardial fibers. Given the restriction that the whole heart cannot always provide an adequate experimental model for many of the approaches required, and the situation that certain re-entrant pathways in the heart occur as a result of damage that occurs to myocardial contractile tissue induced by arrhythmogenic agents or ischemia, the isolated adult cardiomyocyte can offer advantages not available using the adult heart.

The antiarrhythmic properties of n-3 PUFAs have been studied acutely using both neonatal rat cardiomyocyte preparations[1,98–103] and adult rat ventricular cardiomyocytes.[118–120] Malignant arrhythmias (asynchronous contractile activity) can be induced by a variety of chemical stimuli such as β-adrenergic receptor stimulation with isoproterenol, the membrane perturbent lysophosphatidylcholine, elevated extracellular calcium, or ouabain treatment. The n-3 PUFAs, and to a lesser extent, the n-6 PUFAs, but not saturated fatty acids, provide a protective effect against arrhythmias induced by exposure to the above arrhythmogens. It has been reported that acutely added PUFAs are required to be in their free acid form or in the form of a salt and are ineffective when added as ethyl esters. Furthermore, incorporation of fatty acids into the phospholipids of the sarcolemmal membrane was reported not to be required for the fatty acids to produce their antiarrhythmic effects in the neonatal cardiomyocyte model.[121] It has been suggested that n-3 PUFA enrichment of cardiomyocyte membrane lipids leads to a reduced degradation of membrane phospholipids via phospholipase action,[122] although this is in contrast to the results reported by Malis et al.[123] If there is a reduced degradation of phospholipids, this may benefit cell membrane stability and provide protection during episodes of hypoxia and reoxygenation. Furthermore, the type of nonesterified fatty acid released following hydrolysis of membrane phospholipids may determine the nature of the arrhythmic response of the myocardium.[124] If enhanced release of n-3 PUFAs occurs,[123] it may allow a greater reserve of free fatty acids to act in an antiarrhythmic manner if indeed the antiarrhythmic effect requires the n-3 PUFAs to be in the nonesterified form. In neonatal cardiomyocytes, irregularities in the spontaneous contractile frequency were reduced significantly following 4 days of culture in medium supplemented with n-3 PUFAs. An increase in membrane phospholipid n-3 PUFA content also was observed after this treatment. This is indicative that at least some components of the contractile cycle that rely on cell automaticity are altered following incorporation of n-3 PUFAs into cultured cardiomyocyte membrane phospholipids.[125]

Cardiomyocyte models of reperfusion injury

Reperfusion injury induced by a free radical generating system

ROS have been shown to be generated during reperfusion following myocardial ischemia in a number of experimental models.[29,32,49–52] In isolated cells, the addition of various free radical generating systems (FRGS) to simulate reperfusion injury caused contractile dysfunction, including arrhythmias, cessation of contractility, and hypercontracture.[50,126–130] In response to a FRGS consisting of purine, XO and iron-loaded transferrin which generates $O_2 \cdot^-$, H_2O_2, and $\cdot OH$, neonatal rat ventricular cardiomyocytes exhibited a decrease in the number of Ca^{2+} transients with eventual cessation of these transients.[131] Further, the cells developed fibrillatory activity followed by a progressive rise in intracellular Ca^{2+} from nanomolar to micromolar levels. This latter event was also associated with blebbing of the cell membrane and hypercontracture. In cardiomyocytes treated with α-tocopherol (18–24 hours preincubation), the Ca^{2+} transients (and associated spontaneous contractions) remained more stable and exhibited a regular rhythm.[131] Reperfusion with normal buffer (i.e., without the FRGS) restored contractile activity and Ca^{2+} transient activity in α-tocopherol-treated cells. However, in cells not treated with α-tocopherol, reperfusion did not reverse the increase in intracellular Ca^{2+}. Similarly, fetal mouse myocytes exposed to extracellularly generated $O_2 \cdot^-$, H_2O_2, and $\cdot OH$ exhibited a cessation of spontaneous contractile activity.[127] Electrical field stimulation temporarily restored contractile activity until cardiomyocytes developed membrane blebs and hypercontraction that could not be reversed by changing to ROS-free medium. Cessation of contractile activity was not associated with an increase in intracellular calcium levels, however, hypercontraction occurred when intracellular calcium levels increased. Thus, hypercontracture, but

not contractile impairment could be attributed to elevated $[Ca^{2+}]_i$.

Pretreatment for 18 hours with α-tocopherol protected against the loss of contractile activity that occurred during exposure to $O_2 \cdot^-$, H_2O_2, and · OH and resulted in decreased lactate dehydrogenase (LDH) release and conjugated diene formation, and decreased [^{3}H] arachidonate release in neonatal rat cardiomyocytes.[128] Trolox [the aromatized polar (hydrophilic) region of the α-tocopherol molecule] and phytol (the hydrophobic tail of α-tocopherol molecule) were not as effective as α-tocopherol per se, indicating that the combined hydrophilic and hydrophobic regions are required for the full antioxidant potential of α-tocopherol to be observed. Other studies have investigated the protection afforded by synthetic antioxidants in reperfusion injury. The SOD mimetic 4-hydroxy-2,2,6,6-tetramethyl-piperidinoxyl (TEMPOL) prevented LDH release and the loss of contractile activity induced by HX/XO, which generates $O_2 \cdot^-$ and H_2O_2.[126] CAT but not SOD or TEMPOL prevented ATP depletion and LDH release, indicating that H_2O_2 is the species predominantly responsible for membrane damage caused by HX/XO whereas $O_2 \cdot^-$ appears to cause relatively less damage. The above also indicates that the protection afforded by TEMPOL may be due to its ability to render H_2O_2 inactive, although even catalase did not totally inhibit LDH release, indicating that other ROS may contribute to damage. The finding that deferoxamine (a cell permeable iron chelator) was partially protective indicates that transition metal ions are involved in the damage caused by HX/XO because H_2O_2 can react with intracellular iron to form · OH. Further support for the involvement of intracellular transition metal ions in reperfusion injury comes from the study of Byler et al.[132] in which it was demonstrated that the cardiomyocyte injury caused by H_2O_2 was not due to the H_2O_2 per se but to toxic radicals (e.g., · OH) formed indirectly from H_2O_2 via iron-catalyzed reactions. Both pretreatment or co-administration of deferoxamine with H_2O_2 significantly reduced LDH release. Iron-loaded deferoxamine was completely ineffective, indicating that the protection afforded by deferoxamine could be attributed entirely to its iron-chelating capacity. Quercetin, which is a naturally occurring flavonoid, has also been reported to provide protection against free-radical induced toxicity through its iron-chelating effects,[57] although this is yet to be demonstrated in isolated heart cells.

Reperfusion injury induced by hypoxia/reoxygenation

In neonatal rat ventricular cardiomyocytes exposed to 2, 4, or 6 hours of hypoxia followed by 1, 2, or 3 hours of reoxygenation, a progressively greater release of LDH occurred with increasing hypoxic time, indicating damage to cardiomyocytes. A similar increase in LDH release was observed following increasing reoxygenation duration, which could be decreased by preincubation with SOD.[133] Although the authors did not document morphologic changes to the cardiomyocytes following hypoxia/reoxygenation, they found that membrane fluidity was decreased after hypoxia/reoxygenation. Because ROS would have been generated intracellularly using the above protocol, it is interesting that SOD, which is unlikely to permeate the cell membrane, was protective. However, because SOD was able to prevent the fluidity changes induced by hypoxia/reoxygenation, a role for extracellular ROS in the membrane damage could be inferred. Protection against hypoxia/reoxygenation induced LDH release in neonatal rat ventricular cardiomyocytes was also found following incubation with α-tocopherol and β-carotene for 6 days.[134]

Vanden Hoek et al.[135] reported that embryonic chick ventricular cardiomyocytes released significantly less LDH during sustained ischemia (4 hours) than during ischemia (1 hour) followed by reperfusion (3 hours). They also reported that increasing the duration of the ischemic period (from 30–90 minutes) increased the extent of injury after 5 hours of reperfusion. This is in contrast to the data of Jennings et al.[136] who argued that the injury at the time of reperfusion merely represents an acceleration of the damage that normally would have occurred. If so, it may be expected that protective pharmacologic agents administered during the reperfusion phase should be capable of limiting tissue necrosis.[27] This was not the case in the study of Vanden Hoek et al.[135] because the metal chelator 1,10-phenanthroline that was present only during reperfusion was not shown to be protective. The presence of this metal chelator during ischemia did not significantly decrease cell death during ischemia, however, after 3 hours of reperfusion, cell death was significantly lower compared with untreated cells. In one study that used fluorescent probes to identify the ROS produced during ischemia and reperfusion in chick cardiomyoctes, during ischemia the oxidation of both 2′7′-dichlorofluorescein diacetate (DCF; oxidized by H_2O_2 and · OH) and dihydroethidium (DHE; oxidized by $O_2 \cdot^-$ and · OH) increased.[137] However, upon reperfusion, although DHE fluorescence levels fell rapidly, DCF fluorescence increased quickly within the first 5 minutes of reperfusion, indicating the presence of H_2O_2 and · OH during the early reperfusion phase. Correspondingly, the use of 1,10-phenanthroline and mercaptopropylene glycol (a synthetic analog of glutathione) throughout ischemia and reperfusion significantly reduced cell death during reperfusion in chick cardiomyocytes. Significant protection against hypoxia/reoxygenation induced injury was demonstrated in cardiomyocytes isolated from rats administered dietary EPA or DHA for 4 weeks.[138] These effects were accompanied by an elevation in the proportion of EPA but not DHA in the myocardial membrane phospholipids. Because reoxygenation following anoxic conditions provokes oscillations in cytosolic Ca^{2+},[139] the protective effects of n-3 PUFAs may be implicated in part in reoxygenation-induced hypercontracture by preventing oscillations of intracellular Ca^{2+} and concomitant free radical production during the early phase of reoxygenation as indicated by the mechanism proposed by Obata et al.[30]

Other mechanisms underlying protective effects on cardiac arrhythmia

Eicosanoids and antiarrhythmic mechanisms

The 20 carbon PUFAs, AA, and EPA, which preferentially occupy the *sn*-2 position of cell membrane phospholipids,

can be released by the action of phospholipase A_2 and metabolized to form the eicosanoids.[40,140,141] AA is metabolized by cyclooxygenase to form the 2-series eicosanoids thromboxane A_2 (TXA_2) and prostaglandin I_2 (PGI_2) (which are involved in inflammation and immune responses[142]) and the 4-series leukotrienes by lipoxygenase, whereas from EPA, the 3-series eicosanoids TXA_3 and PGI_3 are synthesized. The types and amounts of eicosanoids synthesized are determined primarily by the availability of the respective precursors, the activities of those enzymes that release the esterified PUFAs, the activities of cyclooxygenase and lipoxygenases, and the nature of the stimulus.[142] When the proportion of EPA and DHA in the membrane is increased, such as by dietary means, there is concomitantly less AA available to form the 2-series eicosanoids.[141] EPA also competes with AA for the cyclooxygenase enzyme to synthesize the 3-series eicosanoids. In contrast to TXA_2, which has potent vasoconstriction and platelet aggregatory actions, TXA_3 possesses only weak biological properties.[141] Conversely, PGI_3 has vasodilator properties similar to PGI_2. The net result of increased n-3 fatty acids in cell membranes is a change in the hemostatic balance toward one of greater vasodilatation and less platelet aggregation.[40] Studies by Abeywardena and Charnock[39] using rats demonstrated that dietary fish oil feeding reduced the incidence of ventricular arrhythmias, which was attributed to decreased myocardial TXA_2 synthesis and an elevated PGI_2/TXA_2 ratio. It is likely that the shift toward the synthesis of 3-series eicosanoids may be favorable for prevention of cardiovascular disease.

During posthypoxic reoxygenation of neonatal rat cardiomyocytes, the production of eicosanoids is in part dependent on the cell membrane phospholipid n-3 PUFA content.[143] Both PGI_2 and TXA_2 synthesis is reported to be increased during myocardial ischemia and reperfusion.[144] TXA_2 has been shown to increase inositol(1,4,5)-trisphosphate (IP_3) production in neonatal rat cardiomyocytes by approximately 14-fold, and this was likely due to activation of phospholipase C activity.[145] Dietary supplementation with n-3 PUFAs decreases the production of the biologically active thromboxane TXA_2 in favor of TXA_3,[146] and therefore decreased thromboxane production by fish oil supplementation may play a role in decreased IP_3 release during reperfusion. Under conditions of normoxia and reoxygenation, the production of the 2-series prostaglandins was lower in n-3 PUFA supplemented cardiomyocytes than in cells supplemented with n-6 PUFA medium.[143] The effects of the cyclooxygenase and lipoxygenase metabolites of AA and EPA on the activity of spontaneously contracting cultured neonatal rat cardiomyocytes were determined by Li et al.,[147] who reported that changes occurred in both the contraction amplitude and the beat rate of cultured cardiomyocytes following acute addition of the AA metabolites PGD_2, PGE_2, $PGF_{2\alpha}$, or the compound U46619 (a thromboxane mimetic). Superfusion of neonatal cardiomyocytes with low concentrations of the above compounds resulted in the rapid development of tachyarrhythmias, initially characterized by a regular fast rhythm with a reduction in contraction amplitude and chaotic fibrillatory contractile activity occurring at higher concentrations. The 3-series cyclooxygenase metabolites of EPA (PGD_3 and PGE_3) were less potent than the 2-series cyclooxygenase products PGE_2 and $PGF_{2\alpha}$. Furthermore, PGI_2 produced a marked reduction in the beat rate and terminated the tachyarrhythmias induced by $PGF_{2\alpha}$ or U46619. Leukotrienes were reported not to influence the contractile activity of neonatal cardiomyocytes. Eicosanoid-induced arrhythmias have been reported to be terminated by acute addition of EPA or AA plus the cyclooxygenase inhibitor indomethacin.[147] These results, although not directly related to dietary effects, may provide insight as to how the balance of n-3/n-6 PUFAs could impact on the eicosanoid status of the cardiomyocyte and underlie the proarrhythmic or antiarrhythmic effects of these PUFAs.

Effects of fatty acids on cardiac electrophysiology

Cardioprotective properties of dietary fatty acids may be due to modulation of the cardiac action potential, with certain fatty acids acting as membrane stabilizing agents to slow the rate of the upstroke velocity of the action potential.[78,102] The degree of opening of the fast Na^+ channel and hence the initiation of the action potential is voltage dependent and influenced by the extent and rate of prior depolarization.[97] Certain antiarrhythmic drugs (e.g., quinidine), which cause partial depolarization of the membrane, can slow the recovery of the ability of the Na^+ channels to reopen. Evidence obtained from measurement of Na^+ currents in patch-clamped neonatal cardiomyocytes[102,148] and in adult rat cardiomyocytes (Leifert et al., unpublished observations) indicate that the n-3 fatty acids may act in a similar manner. Increasing the extracellular K^+ concentration $[K^+]_o$ lowers the resting membrane potential and causes the cardiomyocyte to be partially depolarized during diastole. Because EPA has been reported to alter the properties of neonatal cardiomyocytes in a similar manner,[102] n-3 PUFAs may modulate the membrane excitability by altering the $[K^+]_i$:$[K^+]_o$ ratio by direct interaction with certain cardiac K^+ channels such as the ATP-dependent K^+ channels (K_{ATP}) during ischemia or, alternatively, indirectly via protein kinase C activation.[149] The underlying mechanism is possibly related to the inactivation of Na^+ channels[148] and these effects of PUFAs on Na^+ current may be important with regard to the antiarrhythmic effects of the n-3 PUFAs.

A number of studies have demonstrated that acute addition of the n-3 PUFAs alters the automaticity of spontaneously-contracting, neonatal cardiomyocytes maintained in culture.[98–100,102,148,150–153] Application of 5 to 15 μM EPA or DHA during superfusion of neonatal cardiomyocytes markedly reduced the contraction rate of the rhythmically, spontaneously contracting syncytia. Furthermore, these n-3 PUFAs both prevented and terminated tachyarrhythmias induced by various arrhythmogens. Although a marked reduction in beat rate occurred, there was no significant change in systolic or diastolic $[Ca^{2+}]_i$. In contrast, verapamil (L-type Ca^{2+} channel blocker) did not slow the beat rate but induced a progressive decline in the amplitude of contractions and Ca^{2+} transients, both of which finally ceased, indicating that acute n-3 PUFA addition did not mimic the action of Ca^{2+} channel blockers. Similar results have been obtained using adult rat ventric-

ular cardiomyocytes.[118–120] Unlike neonatal cardiomyocytes, adult rat cardiomyocytes do not spontaneously contract but remain quiescent. Electrical field stimulation depolarizes the cardiomyocyte sarcolemmal membrane, initiating contractions in synchrony with the applied electrical stimulus. When arrhythmogenic agents such as isoproterenol (a β-adrenergic receptor agonist) are added to the superfusing buffer during electrical field stimulation, asynchronous contractile activity develops. The addition of micromolar concentrations of EPA or DHA (as the free acids) to the superfusing medium after the development of asynchronous contractile activity terminates the electrically-driven contractile activity. Upon increasing the applied voltage, which previously was held just above the threshold level needed to initiate contractile activity, the cardiomyocytes recommence continued synchronous contractions even in the presence of the arrhythmogenic agent. This suggests that the addition of EPA or DHA had some influence on cardiomyocyte sarcolemmal membrane excitability. Similarly, when EPA and DHA were superfused over cardiomyocytes prior to the addition of isoproterenol, EPA and DHA also prevented such asynchronous contractile activity at a suprathreshold voltage. The effect of the β-adrenergic receptor antagonist propranolol (an antiarrhythmic drug) was similar to that of the calcium channel antagonists and quite distinct from that of the n-3 PUFAs. EPA was shown to protect neonatal cardiomyocytes against arrhythmias induced by a Ca^{2+} ionophore even when intracellular Ca^{2+} levels were maintained at relatively high levels by the experimental conditions.[100] Modulation of cardiac contractility may be mediated via alterations in Ca^{2+} cycling within the cardiomyocyte or via changes in the sensitivity of the myofilaments.[154] Alterations in contractile function may also be induced by altering cardiac ion channel activity. AA has been shown to increase the amplitude of the Ca^{2+} transient, which induces a twofold increase in cell shortening when added to spontaneously contracting neonatal cardiomyocytes.[154] Therefore, release of AA by phospholipase action in response to receptor activation by endogenous mediators or pathologic stimuli may be involved in mediating inotropic responses in the myocardium.

The electrophysiologic mechanisms underlying the effects of EPA and DHA are likely to involve changes in automaticity or excitability of cardiomyocytes, which may be induced by changes in the physical state of the sarcolemmal membrane lipids, thus affecting one or more of the five phases of the action potential. The mobility and conformation of intrinsic cell membrane proteins, and thus their function as receptors, enzymes, and ion channels, can be significantly influenced by the physical state of their surrounding membrane lipid environment.[36,122,125,155–157] EPA and DHA have been reported to alter Na^+ channel activity (i.e., voltage/current dependency) in neonatal[148] and adult (Leifert et al., unpublished observations) rat cardiomyocytes. Such a result is consistent with these n-3 PUFAs changing sarcolemmal membrane lipid physical properties, because Na^+ channel activity has been shown to be modulated by changes in its immediate lipid environment (Leifert et al., unpublished observations). Benzyl alcohol, a membrane fluidizing agent,[158] has been reported to exhibit protective effects against isoproterenol-induced asynchronous contractile activity in electrically-stimulated adult rat cardiomyocytes in a manner similar to that for acutely added EPA and DHA.[118–120] Addition of 10 mM benzyl alcohol to the superfusing medium following the development of asynchronous contractile activity in adult rat cardiomyocytes by isoproterenol quickly restored synchronous contractile activity with an underlying requirement for an increase in the voltage of the applied electrical field stimulation, indicative of a change in the threshold voltage required for depolarization. Because fatty acids are able to quickly partition into the membrane bilayer lipids and likely change the threshold voltage for the gating of Na^+ channels that initiate the action potential, it has been suggested that changes in membrane fluidity may be associated with the antiarrhythmic effects of the n-3 PUFAs.[118–120] Indeed, at low concentrations, certain fatty acids display membrane stabilizing effects[36] not unlike the effects of local anesthetics and antiarrhythmic compounds such as lidocaine. Using the technique of steady-state fluorescence anisotropy (SSFA) with the fluidity probe N-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl)trimethyl-ammonium p-toluenesulfonate (TMAP-DPH) changes in the membrane fluidity of adult rat cardiomyocytes were determined following acute addition of various fatty acids.[118–120] SSFA values were unaltered with either the saturated fatty acid, stearic acid (C18:0), behenic acid (C22:0), or the methyl ester form of DHA. In contrast, 10 minutes of incubation with EPA or DHA significantly decreased the SSFA value (r_{ss} value), implying an increase in membrane fluidity.

Fusion of DHA into mouse mitochondrial membranes by DHA incubation increased mitochondrial membrane fluidity as detected using the fluorescent membrane probe DPH,[159] which localizes in the very hydrophobic region of the membrane bilayer.[160] Interestingly, using this same system but with the fluidity probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) which anchors near the bilayer surface due to its charged trimethylammonium head group,[161,162] it was reported that no change in mitochondrial membrane fluidity was apparent.[159] These same mitochondria fused with DHA were shown to have decreased membrane potentials when measured using a membrane potential-sensitive fluorescent probe.[159]

Collectively, these data suggested that PUFAs directly alter the excitability of the cardiac sarcolemmal membrane. Thus, the n-3 PUFAs may prevent asynchronous contractile activity in the isolated cell model and myocardial arrhythmias in vivo by exerting effects on cell excitability, preventing the generation of aberrant action potentials and re-entrant circuits.

Effects of fatty acids on intracellular Ca^{2+} mobilization

Following depolarization, the contractile activity of the myocardium is under the control of $[Ca^{2+}]$, which is controlled by extracellular Ca^{2+} influx into the cardiomyocyte as well as signaled release of Ca^{2+} from intracellular stores, notably the sarcoplasmic reticulum (SR). Relaxation is in part a reversal of these processes although different

enzyme systems and sequestering mechanisms are involved. An enhanced release of the second messenger IP_3 from the sarcolemma has been reported to be associated with the development of ischemic and reperfusion associated ventricular arrhythmias.[163–166] Inhibition of this IP_3 release has been suggested to exert an antiarrhythmic effect.[163] Together these findings would support the notion that the antiarrhythmic effect of the n-3 PUFAs may be related in part to their effects on the activity of the phosphoinositide signaling pathway. Du et al.[165] investigated the effect of fish oil supplementation by gavage on postischemic reperfusion arrhythmias and reported that dietary n-3 PUFA supplementation significantly inhibited both increases in intracellular IP_3 levels and the incidence of reperfusion arrhythmias. Experiments using cultured neonatal cardiomyocytes exposed to DHA for 3 days also support the involvement of the IP_3 pathway in the antiarrhythmic action of DHA.[167] For example, the arrhythmias induced in cardiomyocytes by α_1-adrenoceptor stimulation (which utilizes the phosphoinositide signaling system) were prevented following DHA incubation. In addition, n-3 PUFA pretreatment has been reported to decrease the α_1-adrenoceptor-stimulated formation of IP_3.[125,167] Dietary EPA supplementation in a canine arrhythmia model has been reported to increase the (Ca^{2+}-Mg^{2+})ATPase activity in a myocardial microsomal fraction (presumably enriched in SR membranes).[168] These effects were associated with an increased ratio of EPA to AA within this cellular fraction. Although dietary feeding of fish oil to rats for 21 days has been shown to increase the n-3:n-6 fatty acid ratio in cardiac SR, the cardiac SR membrane associated Ca^{2+}-ATPase activity measured in this study was reduced in those animals fed the fish oil supplemented diet.[169] Therefore, it is likely that the severity of ventricular arrhythmias may be reduced by inhibiting the accumulation of intracellular Ca^{2+} following ischemia by modulating other mechanisms responsible for Ca^{2+} extrusion such as the Na^+/Ca^{2+} exchanger. Preventing the frequency of spontaneous Ca^{2+} release from the SR also may be implicated.[170] Therefore, enrichment of the dietary n-3 PUFA supply, which subsequently elevates the proportions of EPA and DHA in myocardial sarcolemmal and SR membrane phospholipids, may prevent IP_3-induced Ca^{2+} oscillations and the development of subsequent arrhythmias.

Summary and perspectives

In this review, we have attempted to report the results of a number of animal and cellular studies which investigated the possible mechanisms involved in ischemia/reperfusion damage, particularly in the myocardium, and the protective role of n-3 PUFAs and antioxidants. The cardioprotective effects of PUFAs in ischemic arrhythmias have been explained in terms of a number of mechanisms, including competition with arachidonic acid for the cyclooxygenase enzymes, their membrane stabilizing effects through increasing membrane fluidity with concomitant effects on ion channel activity, and, finally, their modulation of intracellular calcium release. In terms of reperfusion injury, the mechanisms alluded to above may still be relevant. However, in view of the large body of evidence supporting the involvement of free radicals in reperfusion injury, an antioxidant-like mechanism for n-3 PUFAs is perhaps more likely, particularly in light of the many studies[70,73,74] that demonstrate the protection of the myocardium from development of arrhythmias during reperfusion. The n-3 PUFAs may be directly or indirectly scavenging free radicals produced at reperfusion or may be acting as free radical sinks following release of fatty acids from the membrane in response to increased $[Ca^{2+}]_i$ or ROS generation.[123] As such, they may be protecting against further membrane damage, although the lipid peroxides formed by oxidation of the n-3 PUFAs could induce further damage. In this context, it is important to note that in both scenarios—ischemia and reperfusion—maximum protection occurs when the n-3 PUFA has been released from membrane phospholipids and is in the nonesterified form. This may optimize the effectiveness of the n-3 PUFAs as cardioprotective agents by reasons that are not yet apparent.

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