

Original Contribution

Dietary fish oil alters cardiomyocyte Ca^{2+} dynamics and antioxidant status

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Abstract

The *n*-3 polyunsaturated fatty acids (PUFAs) found in fish oil (FO) have been shown to protect against reperfusion arrhythmias, a manifestation of reperfusion injury, which is believed to be induced by the formation of reactive oxygen species (ROS) and intracellular calcium (Ca^{2+}) overload. Adult rats fed a diet supplemented with 10% FO had a higher proportion of myocardial *n*-3 PUFAs and increased expression of antioxidant enzymes compared with the saturated fat (SF)-supplemented group. Addition of hydrogen peroxide (H_2O_2) to cardiomyocytes isolated from rats in the SF-supplemented group increased the proportions of cardiomyocytes contracting in an asynchronous manner, increased the rate of Ca^{2+} influx, and increased the diastolic and systolic $[\text{Ca}^{2+}]_i$ compared with the FO group. H_2O_2 exposure increased the membrane fluidity of cardiomyocytes from the FO group. These results demonstrate that dietary FO supplementation is associated with a reduction in the susceptibility of myocytes to ROS-induced injury and this may be related to membrane incorporation of *n*-3 PUFAs, increased antioxidant defenses, changes in cardiomyocyte membrane fluidity, and the ability to prevent rises in cellular Ca^{2+} in response to ROS.

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Free radical-induced oxidative damage has been implicated in the pathogenesis of a number of injury and disease states [1]. While constantly being subjected to oxidative stress, aerobic organisms are normally protected against oxidative damage by a variety of antioxidant systems, including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) [2]. Oxidative damage, however, may occur when antioxidant potential is decreased and/or when oxidative stress is increased. Several lines of evidence support a role for reactive oxygen species (ROS) as mediators of myocardial reperfusion injury [3]. ROS are generated upon restoration

(reperfusion) of blood flow to an ischemic region of the heart [1] and the resulting reperfusion injury encompasses a spectrum of events including arrhythmias and myocardial stunning [4]. In the present study, we exposed adult rat ventricular myocytes to extracellularly generated ROS as a model of the injury caused by ROS generated in the myocardium at the time of reperfusion.

Previous studies in laboratory animals have indicated that dietary fish oil (FO), high in *n*-3 polyunsaturated fatty acids (PUFAs), confers a protective effect from reperfusion-induced arrhythmias [5]. However, some studies have advanced the idea that incorporation of *n*-3 PUFAs into membrane phospholipids after dietary supplementation would bring about damaging effects to cellular membranes by virtue of the susceptibility of the highly unsaturated acyl fatty acids to peroxidation [6,7]. If this heightens the level of oxidative stress, then the antioxidant capacity of the heart may be diminished. However, it is also possible that the *n*-3 PUFA-induced oxidative stress may function to potentiate the defense system and stimulate upregulation of the antioxidant enzymes themselves [8]. Thus one of the aims of the present study was to determine whether dietary fish oil supplementation is associated with increased

Abbreviations: CAT, catalase; $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; CON, control diet; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SF, saturated fat; SOD, superoxide dismutase.

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expression of the antioxidant enzymes manganese-SOD (Mn-SOD), CAT, and GPX.

Dietary lipid supplementation also results in changes in the fatty acid composition and cholesterol content of the cell membranes, which can modify membrane fluidity and thereby alter the function of membrane receptors and membrane-bound enzymes [9,10] and transporters for ions such as sodium and calcium. Intracellular calcium ($[Ca^{2+}]_i$) is an important second-messenger system in the myocardial cycle of excitation–contraction coupling. In cardiac tissue, the elevation of cytosolic Ca^{2+} (which may lead to Ca^{2+} overload) associated with ROS-induced tissue damage during ischemia–reperfusion is linked to various abnormalities such as contractile dysfunction and ventricular arrhythmia [11]. ROS can specifically damage ion pumps, exchangers, and channels, including the Ca^{2+} , sodium, and potassium channels [12]. We hypothesized that the *n*-3 PUFAs may be exerting protective effects by their influence on cellular Ca^{2+} dynamics.

Although reperfusion *in vivo* is preceded by a period of ischemia, the use of a free radical-generating system in the present study was not designed to mimic a cellular state of ischemia and reperfusion but to allow the examination of one component of myocardial damage, that of myocyte contractile dysfunction occurring at reperfusion. Hence we exposed isolated rat cardiomyocytes to ROS as an *in vitro* model of reperfusion injury (ROS-induced injury) and examined the effects of *n*-3 PUFAs after dietary supplementation. We show that adult rat ventricular myocytes challenged with H_2O_2 develop asynchronous contractile activity, which may mimic the arrhythmic contractions of the whole heart [13], these arrhythmias being one of the manifestations of reperfusion injury. Dietary FO supplementation increased *n*-3 PUFA incorporation into myocardial membranes and this was associated with enhanced gene expression of several antioxidant enzymes and reduced cellular Ca^{2+} influx during exposure to ROS. These changes may confer protection from ROS-induced injury.

Materials and methods

Ethics and animal care

Animals were cared for according to the Australian National Health and Medical Council *Guidelines for the Care and Use of Animals*. All experimental procedures were subject to prior approval by the Adelaide University and CSIRO Health Sciences and Nutrition Animal Ethics Committees. Male Sprague–Dawley rats were obtained at 4 weeks of age and fed a low α -linolenic acid reference diet based on the AIN 93G diet, containing 7% (w/w) fat, as Sunola oil. At 9 weeks of age, the animals were given a further 10% (w/w) lipid supplement for 4 weeks. The supplements were either saturated fat (SF; beef and mutton fat) or fish oil (RoPUFA30), providing a total of 17% (by weight) added fat. For the contractility and antioxidant enzyme gene expression studies, a control (CON) group of rats was fed a diet consisting of 17% (w/w) fat present as Sunola oil. For the antioxidant enzyme gene expression studies, the source of fish oil used in the Ca^{2+} studies was no longer available and

therefore the fish oil supplement Fishaphos was used. The fatty acid composition of the ventricular membrane phospholipids was comparable between rats fed the RoPUFA30 and those fed the Fishaphos supplemented diet, hence only the results of the latter are presented. The lipid-supplemented diets were changed every second day to minimize oxidation of the fatty acids. The animals were weighed weekly during the supplementation period.

Fatty acid analysis

Total lipids were extracted from the ventricular tissue, the standard colony diet, and the dietary oils using a modification of the method of Bligh and Dyer [14]. Ventricular tissue (250–300 mg) was homogenized in 1 ml water using a Tenbroeck hand-held homogenizer (10 passes) before lipid extraction. Fatty acid methyl esters were identified using authentic lipid standards (Nu-Chek-Prep, Inc., Elysian, MN, USA) by gas–liquid chromatography. The proportions of the total fatty acids were normalized to a value of 100%.

Isolation of cardiomyocytes

Rat ventricular cardiomyocytes were isolated as described previously [15] and plated onto laminin-coated glass coverslips. Cardiomyocytes were maintained in a humidified incubator at 37°C, gassed with 5% CO_2 in air until use. Cells were used within 30 h of isolation, at which time they still maintained a rod shape with cross-striations clearly visible. All the adhering cardiomyocytes were Ca^{2+} tolerant and did not exhibit spontaneous contractions, contracting only in synchrony with the applied electrical stimulation.

ROS-induced asynchronous contractile activity in cardiomyocytes

Cardiomyocytes attached to glass coverslips were transferred to a custom-designed superfusion chamber (No. 0 glass base) located on the stage of an inverted microscope and superfused at 2.5 ml/min with Tyrode's buffer containing 2 mM Ca^{2+} at 32°C. After 3 min equilibration, cells were stimulated at 0.5 Hz for 2 min, using two platinum wire electrodes placed within the superfusion chamber and connected to a stimulator. Cells were then superfused under these conditions with 30 μ M H_2O_2 , and the development of asynchronous contractile activity was monitored for 20 min. It was found that asynchronous contractile activity could be reproducibly induced under these conditions. In preliminary experiments, within 1 min of exposure to millimolar concentrations of H_2O_2 , cardiomyocytes irreversibly hypercontracted and could not be used for experiments. Thus, rather than employ excessively high concentrations of oxidant, the concentration of H_2O_2 chosen was based on observations from pilot experiments. This concentration (30 μ M) was effective in inducing an oxidative injury and subsequent asynchronous contractile activity in a reasonable time frame for study. The term “asynchronous” was used to

define cells not contracting in synchrony with the applied electrical field stimulation, for example, cells contracting at a rate above or below that of the electrical field stimulation, irregular contractile activity, cells that had ceased contracting, or hypercontracted cells. We have previously induced asynchronous contractile activity in adult rat atrial and ventricular myocytes using a variety of arrhythmogens [15,16].

Measurement of $[Ca^{2+}]_i$ using Fura-2

Isolated cardiomyocytes were loaded with 5 μ M Fura-2 as described previously [17]. After probe loading, the cells were equilibrated as described above and baseline calcium transients were measured. After 2 min, 30 μ M H_2O_2 was introduced into the superfusion chamber and Ca^{2+} transients were measured every 4 min for 20 min, with the actual fluorescence readings being made for a period of 20 s at each time point as described previously. Fluorescence data for the Ca^{2+} transient studies were imported into Acqknowledge software (SDR Clinical Technology, Sydney, Australia) and at least five consecutive Ca^{2+} transients were ensemble averaged to obtain the Ca^{2+} transient parameters for each time point during synchronous contractile activity in the presence of H_2O_2 [17]. During asynchronous contractile activity induced by H_2O_2 , five individual transients were analyzed during the 20-s recording period of the indicated time points and the average measurements were used to obtain the Ca^{2+} transient parameters.

Detection of intracellular ROS with fluorescent probes

Cardiomyocytes plated onto glass coverslips were loaded with 10 μ M 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCFH) for 15 min at 25°C in the dark. Cells were then superfused with Tyrode's solution and equilibrated as described above. CM-DCF fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 535 nm. The difference in the fluorescence reading at baseline and after 20 min superfusion with H_2O_2 was converted to a percentage increase in fluorescence in order to compare differences between intracellular ROS levels in cells from rats fed a FO diet compared to a SF diet.

Measurement of cardiomyocyte membrane fluidity

Membrane fluidity was determined by measuring the steady-state fluorescence anisotropy (R_{ss}) of the probe 3-(diphenylhexatrienyl)propyltrimethylammonium *p*-toluene sulfonate (TMAP-DPH) according to a modification of a method described previously [18]. Relative membrane fluidity is inversely related to the R_{ss} . To investigate the effects of free radicals on cardiomyocyte membrane fluidity, a free radical-generating system of 2.3 mM purine and 7 mU/ml xanthine oxidase, which generates H_2O_2 and $O_2^{\bullet-}$ was used. Use of this system has previously been shown to induce asynchronous contractile activity in cardiomyocytes [15].

RNA isolation

Hearts were perfused with Tyrode's solution and then minced into 15- to 20-mg pieces. Randomly chosen pieces totaling approximately 100 mg (representative of the whole heart) were snap frozen in liquid nitrogen and stored at -80°C . Total RNA was isolated using the RNAzol B reagent based on the method of Chomczynski et al. [19]. The DNA-Free Kit was used to remove contaminating DNA from the RNA preparations. RNA (10 μ g) was electrophoresed in 1.5% (w/v) agarose to confirm the presence of intact RNA.

Real-time RT-PCR

Primers for real-time RT-PCR were designed using the Primer Express Software except for GAPDH, for which previously published primer sequences were used [20]. The primer sequences were as follows: Mn-SOD forward primer, GCCTCCCTGACCTGCCTTAC; reverse primer, GCATGATCTGCGCGTAAATG (GenBank Accession No. NM_017051); catalase forward primer, CCCAGAAGCCTAA-GAATGCAA; reverse primer, GCTTTTCCCTTGGCAGC-TATG (GenBank Accession No. NM_012520); GPX forward primer, GTGTTCCAGTGCGCAGATACA; reverse primer, GGGCTTCTATATCGGGTTCGA (GenBank Accession No. X12367); GAPDH forward primer, ATGTTCCAGTAT-GACTCCACTCACG; reverse primer, GAAGACACCAGTA-GACTCCACGACA. Oligonucleotide primers were synthesized by GensetOligos (Lismore, NSW, Australia). The Applied Biosystems GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA) was used for real-time RT-PCR. PCRs were performed in a 20- μ l volume containing SYBR Green PCR Master Mix (1 \times concentration), 500 nM forward primer, 500 nM reverse primer, and cDNA equivalent to that generated from 20 ng total RNA. A cycle threshold (C_t) was calculated for each sample using the GeneAmp 5700 software [21]. Standard curves were generated for each gene using a reference cDNA containing equal proportions of cDNA isolated from the three diet groups. Standard curves covered a range of cDNA equivalent to that generated from 1 to 200 ng RNA for all genes. Curves were plotted as log starting RNA versus C_t , and expression in each sample, relative to expression in the standard, was calculated from the standard curve. Results were normalized against those obtained for GAPDH mRNA.

Chemicals

DMEM culture medium, BSA (fraction V), carnitine, creatine, taurine, 2,3-butanedione monoxime, protease (type XIV), laminin, purine, and xanthine oxidase were purchased from Sigma Chemical Co. (Castle Hill, NSW, Australia). Collagenase was from Yakult Honsha Co., Ltd. (Tokyo, Japan). Penicillin/streptomycin was from GIBCO BRL (Melbourne, VIC, Australia). Fluorescent probes were purchased from Molecular Probes (Eugene, OR, USA) and the reagents for

Table 1
Fatty acid composition (%) of ventricular total phospholipids from rats after dietary lipid supplementation

FAME ^a	CON	SF	FO
14:0	0.12 ± 0.00	0.21 ± 0.02 ^d	0.23 ± 0.01 ^g
16:0	10.84 ± 0.32	12.22 ± 0.41	13.41 ± 0.51 ^f
16:1	0.20 ± 0.01	0.17 ± 0.02	0.25 ± 0.03
17:0	0.15 ± 0.02	0.40 ± 0.02 ^d	0.40 ± 0.01 ^g
18:0	25.27 ± 0.26	25.15 ± 0.23	23.97 ± 0.39 ^{e,h}
18:1 ^b	12.74 ± 0.40	12.14 ± 0.30	9.28 ± 0.22 ^{g,j}
18:2 <i>n</i> -6	8.57 ± 0.67	8.66 ± 0.34	5.97 ± 0.65 ^{e,h}
18:3 <i>n</i> -3	n/d	n/d	n/d
20:0	0.19 ± 0.01	0.19 ± 0.00	0.19 ± 0.01
20:1	0.13 ± 0.01	n/d	0.13 ± 0.00
20:2	0.22 ± 0.03	0.47 ± 0.02 ^d	0.12 ± 0.00 ^{e,j}
20:3 <i>n</i> -6	0.20 ± 0.02	0.29 ± 0.01 ^c	0.29 ± 0.03 ^c
20:4 <i>n</i> -6	27.42 ± 0.66	26.95 ± 0.37	15.90 ± 0.87 ^{g,j}
22:0	0.38 ± 0.02	0.24 ± 0.01 ^d	0.32 ± 0.02 ^h
22:1	n/d	n/d	0.32 ± 0.01
20:5 <i>n</i> -3	n/d	0.14 ± 0.03	3.32 ± 0.07 ^j
24:0	5.22 ± 0.50	2.06 ± 0.13 ^d	0.30 ± 0.04 ^{g,i}
24:1	n/d	n/d	0.14 ± 0.01
22:5 <i>n</i> -3	0.41 ± 0.03	0.87 ± 0.04 ^d	2.52 ± 0.05 ^{g,j}
22:6 <i>n</i> -3	8.03 ± 0.18	9.90 ± 0.43	23.29 ± 1.21 ^{g,j}
Σ SFA	42.09 ± 0.54	40.48 ± 0.48	38.55 ± 0.87 ^f
Σ MUFA	13.07 ± 0.40	12.33 ± 0.29	10.08 ± 0.21 ^{g,j}
Σ PUFA	44.84 ± 0.56	47.19 ± 0.66	51.37 ± 0.87 ^{g,i}
Σ <i>n</i> -6	36.18 ± 0.43	35.91 ± 0.50	22.16 ± 0.61 ^{g,j}
Σ <i>n</i> -3	8.44 ± 0.20	10.82 ± 0.43	29.13 ± 1.24 ^{g,j}
<i>n</i> -6/ <i>n</i> -3	4.29 ± 0.08	3.35 ± 0.15 ^d	0.77 ± 0.06 ^{g,j}

Data are means ± SEM for six rats. The shorthand notation for fatty acid structure is “*a*:*b* (*n*-*c*)” where *a* represents the total number of carbons in the fatty acyl chain, *b* is the number of double bonds in the chain, and *c* represents the number of carbon atoms between the methyl end of the chain and the first double bond. Computational parameters (Σ and *n*-6/*n*-3) were derived from the full fatty acid set. Abbreviations: CON, control diet (17% Sunola oil); SF, saturated fat diet; FO, fish oil diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n/d, not detectable.

^a FAME, fatty acid methyl esters.

^b 18:1 contains *n*-7 and *n*-9 isomers.

^c *p* < 0.05 for CON vs SF.

^d *p* < 0.001 for CON vs SF.

^e *p* < 0.05 for CON vs FO.

^f *p* < 0.01 for CON vs FO.

^g *p* < 0.001 for CON vs FO.

^h *p* < 0.05 for SF vs FO (by ANOVA with the Bonferroni multiple comparisons test).

ⁱ *p* < 0.01 for SF vs FO (by ANOVA with the Bonferroni multiple comparisons test).

^j *p* < 0.001 for SF vs FO (by ANOVA with the Bonferroni multiple comparisons test).

the molecular biology experiments were purchased from GeneWorks Pty. Ltd. (Adelaide, Australia), Applied Biosystems, or GenSetOligos. All other chemicals were of the highest grade available. BSA was delipidated by washing in acetone, petroleum spirit, and diethyl ether (50 g BSA was washed in 200 ml of each solvent three times, filtered, and vacuum dried).

Statistics

Statistical analyses were performed using the computer software program InStat version 3.0 (GraphPad Software, San

Diego, CA, USA). One-way or two-way analysis of variance (ANOVA) with Bonferroni multiple comparison test was used to compare differences between effects of the dietary treatments. Results are expressed as means ± SEM. For each comparison, the criterion for significance was set at *p* < 0.05.

Results

Animal and cellular parameters

The average weight of the rats in the FO group (482.5 ± 11.6 g, *n* = 10) was not significantly different from that of the SF group (493.1 ± 10.8 g, *n* = 13). Furthermore, the viability of cells isolated from the dietary lipid-supplemented rats was not significantly different between the FO and the SF groups (76.1 ± 1.1%, *n* = 7, and 77.5 ± 1.5%, *n* = 8 rats, respectively) as determined by their rod-shaped morphology, immediately after isolation. Similarly for the antioxidant enzyme gene expression study, the average body weight of the rats in the CON group (500.4 ± 13.8 g) was not significantly different from that of the SF (494.8 ± 10.8 g) or FO (498.5 ± 06.1 g) group.

Myocardial fatty acids

The fatty acid compositions of myocardial total phospholipids from rats fed the lipid-supplemented diets are shown in Table 1. Dietary lipid supplementation resulted in significant changes to the fatty acid profile. In comparison with the CON and SF treatment, dietary FO resulted in an increase in the total *n*-3 PUFAs as a result of significantly increased proportions of docosahexaenoic acid (22:6, *n*-3), docosapentaenoic acid (22:5, *n*-3), and eicosapentaenoic acid (20:5, *n*-3). Concomitantly, the proportion of the total *n*-6 PUFAs in the FO-supplemented rats was reduced as a result of the decrease in the proportions of arachidonic acid (20:4, *n*-6) and linoleic acid (18:2, *n*-6). Collectively, this resulted in a significant reduction in the *n*-6/*n*-3 PUFA ratio in the FO-fed rats (*p* < 0.001 vs CON and SF). The overall proportion of saturated fatty acids was reduced in both of the dietary lipid-supplemented groups but reached

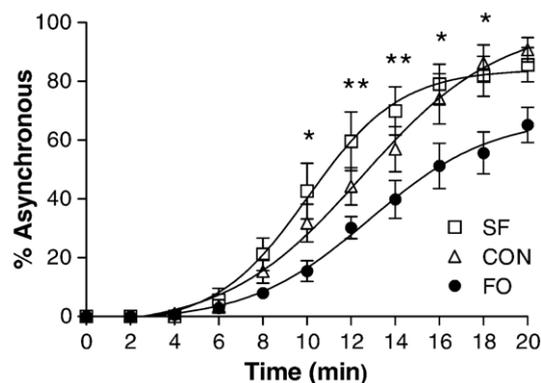


Fig. 1. Development of asynchronous contractile activity after addition of 30 μM H₂O₂. Data are shown as the means ± SEM with each point being the average of approximately 60 cells from a total of three coverslips, for *n* = 4–6 rats. Abbreviations used: CON, control; FO, fish oil; SF, saturated fat diet. **p* < 0.05; ***p* < 0.01 (ANOVA).

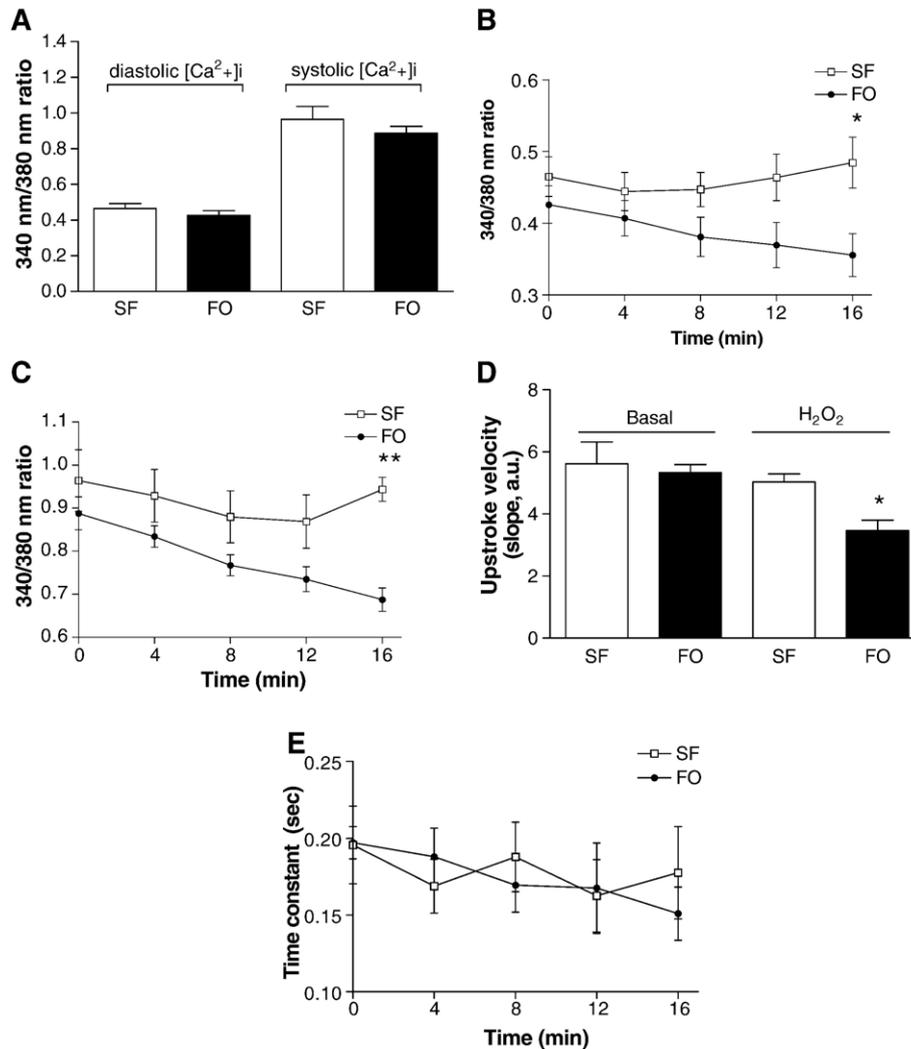


Fig. 2. Calcium handling and ROS. Cells were isolated from rats fed SF- or FO-supplemented diets for 4 weeks and loaded with 5 μ M Fura-2. Cells were stimulated at 0.5 Hz in Tyrode's buffer containing 2 mM Ca^{2+} at 32°C. Data are presented as means \pm SEM of the 340/380 nm Fura-2 ratio for three or four cells from each of five or six rats. (A) Diastolic and systolic $[\text{Ca}^{2+}]_i$ of cardiomyocytes under resting cell conditions. Changes in (B) diastolic and (C) systolic $[\text{Ca}^{2+}]_i$ during H_2O_2 superfusion in myocytes from the SF and FO dietary groups are shown. 30 μ M H_2O_2 was added at time 0. A large proportion of cells developed asynchronous contractile activity after 16 min, hence only the results up to 16 min are presented. (D) The rate of Ca^{2+} influx (upstroke velocity) at rest (basal) and after exposure to H_2O_2 in cells isolated from the SF and FO dietary groups. (E) The effect of 30 μ M H_2O_2 on the time constant of Ca^{2+} transient decay. Abbreviations used: FO, fish oil-supplemented diet; SF, saturated fat-supplemented diet. * $p < 0.05$; ** $p < 0.01$ (ANOVA for SF vs FO at 16 min). Tyrode's buffer contained (in mM): 137.7 NaCl, 4.8 KCl, 1.2 KH_2PO_4 , 1.2 MgSO_4 , 11 glucose, 10.0 *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid).

statistical significance only in the FO group ($p < 0.01$ vs CON). The other significant change in the FO-supplemented rats was the lowering of the proportion of total monounsaturated fatty acids ($p < 0.001$ vs CON and SF) due mainly to the lowered proportion of oleic acid (18:1) in the cardiac membrane phospholipids. In both dietary groups, the proportions of α -linolenic acid (18:3, *n*-3) were below the limits of detection.

Free radical-induced asynchronous contractile activity of cardiomyocytes

Cardiomyocytes electrically stimulated at 0.5 Hz developed asynchronous contractile activity upon superfusion with 30 μ M H_2O_2 at 32°C. As shown in Fig. 1, FO feeding significantly shifted the asynchrony curve to the right compared to the response of cells from the CON group, indicating that the cells

from the FO-supplemented rats were less sensitive to the effects of exogenous H_2O_2 with comparatively less damage occurring over time. After 20 min superfusion with H_2O_2 , $85.7 \pm 5.8\%$ of cells (approx 20 cells from each of five rats) from the SF group were contracting in an asynchronous manner compared with $90.8 \pm 4.2\%$ (approx 20 cells from each of six rats) for the CON group and $65.2 \pm 6.0\%$ (approx 20 cells from each of four rats) in the FO group. At all time points between 10 and 18 min after the addition of H_2O_2 , there was a consistently lower percentage of cells from the FO group contracting in an asynchronous manner compared to SF cells ($p < 0.05$).

Ca^{2+} handling, fish oil, and H_2O_2

The baseline diastolic and systolic $[\text{Ca}^{2+}]_i$ levels were not significantly different between cells isolated from animals in

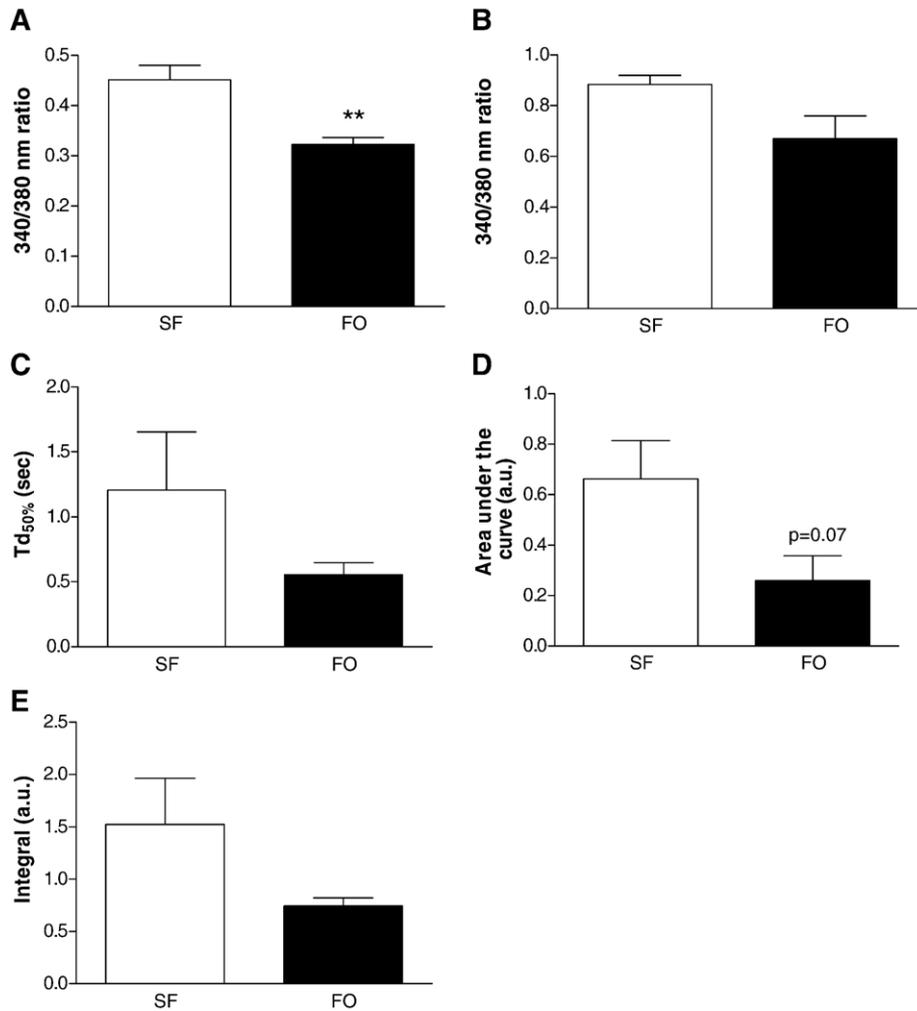


Fig. 3. Calcium handling during asynchronous contractility. (A) Diastolic and (B) systolic $[Ca^{2+}]_i$ in asynchronously contracting SF and FO cells. Changes in (C) $T_{d50\%}$, (D) area under the Ca^{2+} transient, and (E) integral of the Ca^{2+} transient, during H_2O_2 superfusion, for cells isolated from rats fed SF or FO diets are presented. Ratios were measured 20 min after $30 \mu M H_2O_2$ addition. Loading and superfusion conditions were as presented in the legend to Fig. 2. Data are presented as means \pm SEM for $n = 3$ or 4 cells from each of five or six rats per dietary group. ** $p < 0.01$.

the SF and in the FO dietary groups while being electrically stimulated at 0.5 Hz (Fig. 2A). During superfusion with H_2O_2 , the 340/380 nm Fura-2 ratio in the SF group gradually increased, reflecting an increase in the diastolic $[Ca^{2+}]_i$, whereas the diastolic $[Ca^{2+}]_i$ in the FO group decreased (Fig. 2B). Hence, at 16 min after the addition of H_2O_2 the diastolic $[Ca^{2+}]_i$ of the FO group was 36% lower than in the SF group ($p < 0.05$). Similarly, after 16 min superfusion with H_2O_2 , the systolic $[Ca^{2+}]_i$ for cells from the FO group was 37% lower than that from the SF group ($p < 0.01$, Fig. 2C). Only cells that were contracting in a synchronous manner during this period were analyzed for cell $[Ca^{2+}]_i$.

The rate of Ca^{2+} influx into the cytoplasm was measured as the slope of the upstroke of the ensemble averaged Ca^{2+} transients. The basal slope (in the absence of H_2O_2 , expressed as arbitrary units) was not significantly different between cells in the FO and in the SF group (Fig. 2D). However, after exposure to $30 \mu M H_2O_2$, the rate of influx of Ca^{2+} into the FO cells ($n = 6$ rats) was 31% lower compared to the SF group ($n = 5$ rats; $p < 0.05$). There was no significant difference

between the cells from the two dietary groups, with regard to the time constant of Ca^{2+} decay (indicating the rate of Ca^{2+} removal from the cytoplasm, i.e., cell relaxation) either before

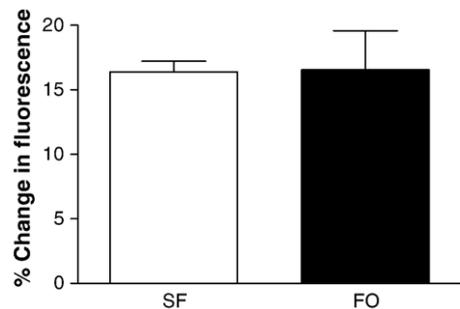


Fig. 4. Change in fluorescence intensity of CM-DCF after $30 \mu M H_2O_2$ addition. Cardiomyocytes were loaded with $10 \mu M$ CM-DCFH for 15 min at room temperature in the dark followed by exposure to $30 \mu M H_2O_2$ for 20 min at $32^\circ C$. CM-DCF fluorescence intensity was measured at an excitation wavelength of 490 nm and an emission wavelength of 535 nm. Fluorescence is presented as the mean \pm SEM for $n = 3$ to 4 cells from each of six rats for both the FO (fish oil) and the SF (saturated fat) dietary groups.

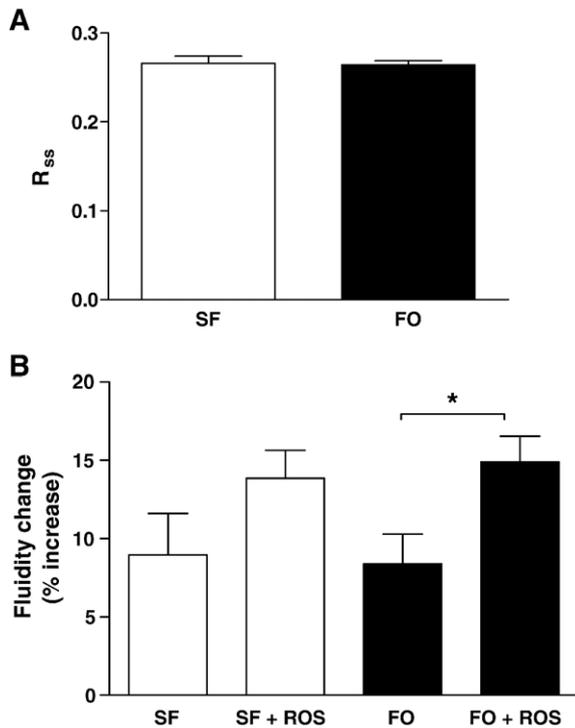


Fig. 5. Cardiomyocyte membrane fluidity of cells isolated from SF- or FO-fed rats. (A) Fluidity under basal conditions. (B) Cardiomyocyte membrane fluidity after ROS addition. Cardiomyocytes were loaded with 2 μ M TMAP-DPH for 15 min and R_{ss} values measured. Purine and xanthine oxidase were added and measurements were taken every 5 min for a total of 20 min. The percentage change in fluidity (relative to the original (baseline) R_{ss} value) at each time point was determined and the change in fluidity after 20 min was plotted for cells isolated from both dietary groups. Abbreviations used: SF, saturated fat diet; FO, fish oil diet; ROS, reactive oxygen species. * $p < 0.05$ for FO vs FO + ROS. FO: $n = 8$ rats, 3 coverslips per rat. SF: $n = 6$ rats, 3 coverslips per rat.

or after the addition of 30 μ M H_2O_2 (Fig. 2E). However, within the FO group itself, after 16 min of exposure to H_2O_2 , there was a significant decrease in the value of the time constant ($p < 0.05$ vs basal, Fig. 2E).

Ca²⁺ handling during asynchronous contractile activity

The diastolic $[Ca^{2+}]_i$ in SF cells was 28% higher than in the FO group during asynchronous contractile activity ($p < 0.01$; Fig. 3A). There was a trend (which did not reach significance) toward a higher systolic $[Ca^{2+}]_i$ in the SF group, which had a value 24% higher than that of the FO group (Fig. 3B). The time to 50% decay of the Ca^{2+} transient ratio ($T_{d50\%}$) was used as a measure of the size of the Ca^{2+} transients during asynchronous contractile activity, with a longer $T_{d50\%}$ indicating a broader transient and likely representing greater Ca^{2+} cycling due to the delayed removal of Ca^{2+} from the cell. Indeed, the “wider” transients corresponded to asynchronous contractile activity. The $T_{d50\%}$ value was lower in the FO cells at 20 min after the addition of H_2O_2 , i.e., a less broad transient was observed, which would suggest a reduced risk of Ca^{2+} overload; however, this change did not reach statistical significance (Fig. 3C). Neither the area under the curve (Fig. 3D) nor the integral (Fig. 3E) was significantly different between the SF and the FO

groups during asynchronous contractile activity. However, there was a trend toward a lower area and integral in the FO group suggestive of reduced Ca^{2+} cycling within the cells. It is possible that statistical significance was not achieved due to the large variation in the data obtained from cells isolated from the SF-fed animals.

Measurements of intracellular ROS production by fluorescence microscopy

The basal levels of ROS in cells from the SF group were not significantly different from those of the FO group, with the fluorescence intensity of CM-DCF in SF cells being 1.28 ± 0.17 (absorbance units) ($n = 6$ rats) compared with 0.92 ± 0.07 ($n = 6$ rats) for the FO cells. After superfusion with 30 μ M H_2O_2 , the fluorescence of CM-DCF increased over the 20-min period of the study. However, the percentage increase in fluorescence intensity 20 min after addition of H_2O_2 was not significantly different between cells derived from the SF- and FO-supplemented rats (Fig. 4).

Membrane fluidity

The membrane fluidity of cardiomyocytes isolated from rats fed a FO supplemented diet was not significantly different from that of cells isolated from rats fed the SF diet (Fig. 5A). It was noted upon probe addition under basal conditions (no ROS present) that the relative fluidity (i.e., the R_{ss} reading) in the untreated cells decreased significantly during the first 20 min in cells isolated from both the SF- and the FO-fed rats ($p < 0.05$ and $p < 0.01$, respectively), indicating a relative increase in cell membrane fluidity over time (results not shown). Hence, when investigating the effect of dietary lipids on ROS-induced changes in fluidity, the changes observed above this gradual increase in membrane fluidity were calculated. In the SF group there was a further 4.9% increase in fluidity after 20 min incubation with ROS, compared to untreated cells (no ROS addition); however, this did not reach statistical significance (Fig. 5B). In the FO group, addition of ROS caused a further increase in fluidity of 6.5% ($p < 0.05$). Therefore, whereas the dietary lipid supplementation did not influence the overall membrane fluidity as measured by steady-state fluorescence polarization, treatment of cardiomyocytes with ROS led to an increase in cell membrane fluidity (i.e., a decrease in R_{ss} value), which reached significance only in the FO group.

Table 2
Summary of C_t values after real-time PCR amplification

	CON	SF	FO
Mn-SOD	25.21 \pm 0.33	25.14 \pm 0.34	23.99 \pm 0.23
CAT	24.49 \pm 0.43	24.31 \pm 0.56	23.46 \pm 0.35
GPX	25.57 \pm 0.40	25.20 \pm 0.28	24.81 \pm 0.16
GAPDH	24.13 \pm 0.53	24.62 \pm 0.46	24.23 \pm 0.23

The data are presented as means \pm SEM for $n = 8$ rats. Abbreviations used: CAT, catalase; CON, control diet; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; FO, fish oil; Mn-SOD, manganese-containing superoxide dismutase; SF, saturated fat.

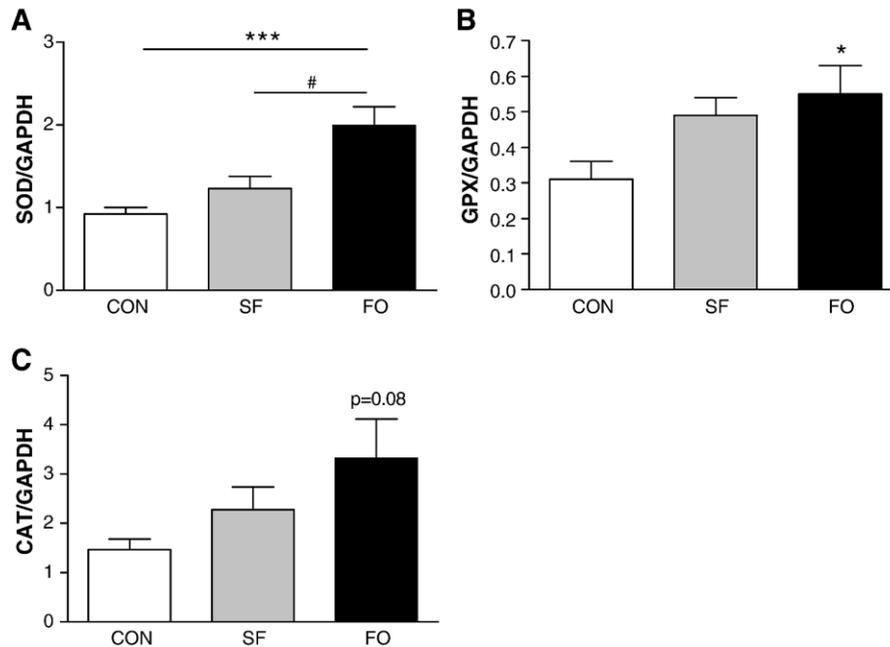


Fig. 6. Expression of antioxidant enzymes in the myocardium of dietary lipid-supplemented rats. Data are normalized to GAPDH for (A) Mn-SOD, (B) CAT, and (C) GPX. Abbreviations used: CAT, catalase; CON, control diet (Sunola oil); FO, fish oil-supplemented diet; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; Mn-SOD, manganese-containing superoxide dismutase; SF, saturated fat-supplemented diet. * $p < 0.05$ for CON vs FO; *** $p < 0.001$ for CON vs FO; # $p < 0.01$ for SF vs FO (ANOVA with the Bonferroni multiple comparisons test), $n = 8$.

Dietary fish oil and antioxidant enzyme gene expression

The average yield of RNA from each rat heart was 0.4 $\mu\text{g}/\text{mg}$ wet wt of heart with an average 260/280 nm ratio of 2.0. Table 2 shows the C_t values obtained after the real-time PCR amplification, before normalization to the housekeeping genes. The relative abundance of mRNA for the antioxidant genes was calculated from the standard curves and expressed relative to the reference cDNA used to construct the standard curve. GAPDH was used as an endogenous control gene, and the relative abundance of GAPDH mRNA was not found to be altered with dietary lipid supplementation. This is consistent with the findings of others who also used GAPDH as an internal control gene in dietary $n-3$ PUFA studies [22,23]. Data are therefore presented as the relative abundance of mRNA for the antioxidant genes, normalized to GAPDH (arbitrary units).

The amount of Mn-SOD mRNA in the FO myocardium was 1.6 times higher than in the SF group ($p < 0.01$) and 2.2 times higher than in the CON group ($p < 0.001$, Fig. 6A). Similarly, the expression of GPX was higher in the FO group ($p < 0.05$ vs CON, Fig. 6B). The expression of CAT was not significantly different between the three lipid-supplemented groups; however, there was a trend toward a higher expression of CAT in the FO group compared to the CON group (Fig. 6C).

Discussion

This report documents the influence of fish oil on ROS-induced injury in rat cardiomyocytes. ROS are believed to be generated at the time of reperfusion after a period of ischemia resulting in reperfusion injury to the myocardium. In the present

study, we exposed adult rat ventricular myocytes to extracellularly generated ROS and demonstrated the development of asynchronous contractile activity in rat cardiomyocytes. Such asynchronous contractile activity may be mimicking the *in vivo* phenomenon of arrhythmia, one of the manifestations of reperfusion injury.

Cells isolated from rats fed a diet supplemented with fish oil incorporated higher proportions of the $n-3$ PUFAs EPA and DHA in their membrane phospholipids and were shown to be less sensitive to the effects of ROS. We determined that the delay in the induction of contractile dysfunction in these (FO) cells was not due to lower concentrations of ROS entering the cells. However, the increased resistance to the effects of ROS in cells isolated from rats fed FO was associated with an increase in cardiomyocyte membrane fluidity after the addition of a free radical-generating system, the attenuation of $[\text{Ca}^{2+}]_i$ levels after exposure to ROS, and enhanced myocardial antioxidant enzyme gene expression after dietary fish oil supplementation.

ROS have been implicated in the pathogenesis of myocardial reperfusion injury possibly via a mechanism that involves damage and perturbation to cell membranes. ROS can alter the chemical and physical properties of cell membranes by modifying the composition, packing, and distribution of the membrane lipids that, in turn, determine fluidity [24]. The present study demonstrated an increase in cardiomyocyte (sarcolemmal) membrane fluidity after ROS addition (which was significant only in the FO group). This is in contrast to previous studies reporting a rigidifying effect (decrease in fluidity) of free radicals on membrane phospholipids [25]. A decrease in fluidity induced by ROS has been attributed to the presence of lipid peroxidation products interacting with

membrane components, enhancing cross-linking among the lipid and protein moieties of the membrane, thereby causing restriction in the freedom and mobility of acyl fatty acid chains in the membrane bilayer. Phospholipase A₂ has been shown to be activated during ischemia and other cellular stresses (reviewed in [26]). Therefore in our study, it is possible that in response to the ROS challenge, the action of phospholipase A₂ liberated the *n*-3 PUFAs from the membrane phospholipids of the FO cells and subsequently, these released free fatty acids induced an increase in the membrane fluidity, as has been previously shown to be related to the antiarrhythmic actions of *n*-3 PUFAs [16]. This is also supported by the finding that fish oil feeding per se increases the activity of phospholipase A₂ in rat mitochondria [27]. Increases in membrane fluidity may affect the functioning of ion channels and membrane transporters and may be responsible for some of the protective effects of the FO diet.

Both ROS and metabolic inhibition can increase intracellular Ca²⁺ levels and induce myocardial Ca²⁺ overload, which in turn can trigger hypercontracture, arrhythmias, and other electrophysiological abnormalities [11,28]. We therefore investigated whether a reduced level of Ca²⁺ cycling was occurring within the FO cells during the ROS challenge. Baseline diastolic and systolic [Ca²⁺]_i were not found to be significantly different between cells isolated from FO or SF diet-fed animals. However, after exposure to H₂O₂, [Ca²⁺]_i, as inferred from the fluorescence ratio measurements, was significantly lower in cells isolated from FO-fed rats. This finding may indicate that, whereas under basal conditions FO feeding may not alter Ca²⁺ dynamics, during free radical stress, it prevents cells from overloading with Ca²⁺ by reducing the extent of Ca²⁺ influx, which is consistent with published reports that acute addition of the *n*-3 PUFAs DHA and EPA reduces the availability of Ca²⁺ for uptake and inhibits the Ca²⁺ release mechanism in rat sarcoplasmic reticulum [29]. Neonatal cardiomyocytes incubated for 3–5 days with EPA were also shown to be protected from developing Ca²⁺ overload in response to 0.1 mM ouabain [30]. It was not in the scope of the present study to investigate which ion channels and transporters were affected by the dietary lipid supplementation and ROS; however, there may be a role for either the sarcoplasmic reticulum or sarcolemmal calcium ion transporters in mediating some aspects of the dietary *n*-3 PUFA effects after cellular stress from ROS as they represent the main pathways responsible for raising [Ca²⁺]_i. The contribution of Ca²⁺ buffering [31] was not examined in the present study; however, it is likely that this factor could influence the cellular Ca²⁺ handling, and the diets themselves may affect Ca²⁺ buffering to different extents. It has been shown that 2–4 h exposure of cardiac myocytes to either nontoxic concentrations of H₂O₂ (0.04 mM H₂O₂) or concentrations that induce apoptosis (0.1–0.2 mM) both result in the upregulation of genes associated with detoxification and removal of ROS [32]. Whether 20 min exposure as used in the present studies is sufficient to induce an adaptive response is not clear and would require future investigation.

Fish oils rich in *n*-3 fatty acids are susceptible to oxidation because of their high degree of unsaturation [6,7]. Indeed, studies have demonstrated a greater extent of oxidative stress

in tissues from humans and animals supplemented with *n*-3 PUFAs [33,34]. In contrast, the *n*-3 PUFA EPA inhibited the γ -irradiation-induced ROS accumulation in rat tissues [35]. In the present study, after 20 min of superfusion with H₂O₂, the overall increase in fluorescence intensity of CM-DCFH (indicative of increased intracellular ROS accumulation) was not significantly different between cells isolated from FO-fed rats compared with rats supplemented with SF. Interestingly, the basal ROS levels were also not significantly different. This finding suggests that the polyunsaturated fatty acid diet did not result in a significant alteration to the level of oxidative stress in the cardiomyocytes, which is in accord with other reports [22]. Furthermore, the fact that the intracellular ROS levels did not change after the H₂O₂ challenge, although other parameters such as intracellular calcium and fluidity were altered, is difficult to interpret, particularly in light of the fact that such challenges did affect the extent of asynchronous contractile activity. The protective effect of dietary FO, in the presence of comparable intracellular levels of ROS, could be explained by a scavenging system in the cardiomyocytes isolated from the FO-fed rats which counteracts the damaging effects of free radicals on cellular function to a relatively greater extent. Indeed, it has been suggested that cells are not always passive to increased oxygen radical production and moderate episodes of free radical stress (for example, induced by fish oil feeding) may in fact potentiate the defense system by stimulating the expression of the antioxidant enzymes [8,36]. Consistent with this notion are the reports that FO feeding resulted in increased renal [22] and hepatic [23] expression of the antioxidant enzymes CAT, SOD, and GPX in mice. Furthermore, the present study demonstrates for the first time an increased myocardial expression of SOD and GPX in rats after fish oil feeding. Future studies may benefit from the measurement of antioxidant enzyme gene expression and fatty acid composition in the cardiomyocytes before and immediately after the challenge by ROS. This would enable a direct comparison of the influence of dietary fish oil on these parameters during ROS-induced injury.

Several mechanisms probably contribute to the enhanced gene expression of antioxidant enzymes after *n*-3 PUFA supplementation. The direct effects of the *n*-3 PUFAs on gene expression are likely mediated by their ability to regulate the activity or abundance of transcription factors [37]. For example, it was shown that DHA can activate the retinoic X receptor [38]. It has also been proposed that PUFAs (or a PUFA metabolite) are transferred to the nucleus, where they function as ligands or modifiers of a nuclear fatty acid receptor-binding protein (PUFA-BP). After PUFA-dependent modification of the PUFA-BP, the PUFA-BP interacts with a *cis*-acting element in the target gene that governs transcription of particular genes [39]. The transcription factor nuclear factor κ B (NF- κ B) has been shown to be activated during myocardial reperfusion [40]. FO feeding combined with food restriction has been shown to prolong the life span of autoimmune-prone mice and this was associated with a blunting of the age-dependent increase in NF- κ B concomitant with increased SOD and CAT activity [41]. This finding seems not only to provide support for the non-pro-

oxidative effect of FO, but also to demonstrate its antioxidant-like activity. In view of the many studies demonstrating an increase in activity of antioxidant enzymes after FO feeding, it is likely that the action of fish oil is predominantly at the level of transcription. Indeed it has been shown that PUFAs activate multiple mechanisms to influence hepatic gene transcription (reviewed in [42]). However, fatty acids can also modulate gene expression indirectly by altering the membrane fatty acid composition or by influencing the generation of intracellular lipid second messengers, e.g., diacylglycerol and ceramide [37]. The classic effect of FO feeding is a decrease in the *n*-6 fatty acid arachidonic acid and a concomitant increase in the phospholipid *n*-3 fatty acids EPA and DHA. Once incorporated into the phospholipids, *n*-3 PUFAs can affect a vast array of intracellular signaling pathways leading to altered gene expression [37].

In this study we have shown that dietary FO supplementation raised *n*-3 PUFA proportions in rat myocardium and this was associated with enhanced antioxidant enzyme gene expression. Although indices of oxidative stress were not directly measured in any rat organs or cells, it is unlikely that the FO diet resulted in significant long-term oxidative stress because we were able to isolate viable cells that demonstrated normal contractility and in studies with whole hearts (isolated from animals fed the same diets, results not shown), cardiac contractile function was not compromised by the FO diet. Moreover, if the FO did initially result in oxidative stress, then the myocardium rapidly adapted and increased some of its antioxidant defenses as evidenced in this study. This may have been responsible for the protective effects against exogenously added free radicals that we observed. Finally, we have identified what could be yet another mechanism by which the *n*-3 PUFAs exert their cardioprotective effects—that of strengthening the endogenous myocardial antioxidant defense system.

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