Dietary fish oil is antihypertrophic but does not enhance postischemic myocardial function in female mice

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1Cardiac Phenomics Laboratory, Department of Physiology and 3Murdoch Children’s Research Institute, Department of Paediatrics, University of Melbourne and Department of Cardiology, Royal Children’s Hospital, Parkville, Victoria; 2Department of Physiology, Monash University, Victoria; 4Division of Medical Sciences, Graduate School of Medicine, School of Health Sciences, University of Wollongong, New South Wales, Australia; 4Department of Medicine, University of Lausanne Medical School, Lausanne, Switzerland

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Huggins CE, Curl CL, Patel R, McLennan PL, Theiss ML, Pedrazzini T, Pepe S, Delbridge LM. Dietary fish oil is antihypertrophic but does not enhance postischemic myocardial function in female mice. Am J Physiol Heart Circ Physiol 296: H957–H966, 2009. First published January 30, 2009; doi:10.1152/ajpheart.01151.2008.—Clinically and experimentally, a case for omega-3 polyunsaturated fatty acid (PUFA) cardioprotection in females has not been clearly established. The goal of this study was to investigate whether dietary omega-3 PUFA supplementation could provide ischemic protection in female mice with an underlying genetic predisposition to cardiac hypertrophy. Mature female transgenic mice (TG) with cardiac-specific overexpression of angiotensinogen that develop normotensive cardiac hypertrophy and littermate wild-type (WT) mice were fed a fish oil-derived diet (FO) or PUFA-matched control diet (CTR) for 4 wk. Myocardial membrane lipids, ex vivo cardiac performance (intraventricular balloon) after global no-flow ischemia and reperfusion (15/30 min), and reperfusion arrhythmia incidence were assessed. FO diet suppressed cardiac growth by 5% and 10% in WT and TG, respectively (P < 0.001). The extent of mechanical recovery [rate-pressure product (RPP) = beats/min × mmHg] of FO-fed WT and TG hearts was similar (50 ± 7% vs. 45 ± 12%, 30 min reperfusion), and this was not significantly different from CTR-fed WT or TG. To evaluate whether systemic estrogen was masking a protective effect of the FO diet, the responses of ovariectomized (OVX) WT and TG mice to FO dietary intervention were assessed. The extent of mechanical recovery of FO-fed OVX WT and TG (RPP, 50 ± 4% vs. 64 ± 8%) was not enhanced compared with CTR-fed mice (RPP, 60 ± 11% vs. 80 ± 8%, P = 0.335). Dietary FO did not suppress the incidence of reperfusion arrhythmias in WT or TG hearts (ovary-intact mice or OVX). Our findings indicate a lack of cardioprotective effect of dietary FO in females, determined by assessment of mechanical and arrhythmic activity postischemia in a murine ex vivo heart model. polyunsaturated fatty acids; ischemia-reperfusion; Langendorff perfused hearts

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LARGE CLINICAL TRIALS HAVE demonstrated that when omega-3 polyunsaturated fatty acid (PUFA) from fish oil (FO) [docosahexaenoic acid (DHA) + eicosapentaenoic acid (EPA)] are used in secondary prevention following myocardial infarction, there is a marked reduction in cardiac-related death and particularly in sudden cardiac death (3, 17). More recently omega-3 PUFA supplementation (DHA + EPA) has been demonstrated to be a beneficial adjunct to present therapies for treatment of heart failure (16). Although these findings have been of landmark significance, a limitation has been the underrepresentation of women, with women either not included in the cohort (3) or comprising only a small proportion (8–20%) of patients (16, 17). This limited female representation is significant because sex differences in cardiac function and in the incidence and progression of cardiovascular diseases are well documented (10, 25).

One recent exception is the Japan EPA Lipid Intervention Study (JELIS), which was a large study (18,645 participants) of predominantly postmenopausal women (~70%). Interestingly, this study found no significant association between EPA supplementation and sudden cardiac death (49). It is plausible that this reflects a sex specificity of omega-3 PUFA not previously identified (34). In a subanalysis of the JELIS cohort, a risk reduction for major coronary events (combining sudden cardiac death, fatal myocardial infarction, and nonfatal myocardial infarction) could be identified but only in the quartile comprising a largest ratio of men to women (40).

Experimentally there is also a dearth of information on the cardiac effects of omega-3 PUFA in the female heart. There is some evidence to suggest that the balance between omega-3 and omega-6 PUFA affects males and females differently. Korotkova et al. (26) demonstrated that the supply of dietary PUFA (of varying ratios of n3:n6 PUFA) during the perinatal period in rats had long-term effects on blood pressure in adulthood that were different for males and females (associated with the specific n3:n6 PUFA ratio of the diet). Thus the possibility that dietary omega-3 PUFA supplementation may have distinct cardiovascular actions in females requires evaluation.

There is strong evidence from experimental studies (of males) that dietary omega-3 PUFA have antiarrhythmic properties in the setting of ischemia and reperfusion and postmyocardial infarction (29, 31, 39) although the cellular mechanisms are incompletely understood and controversial (4, 29). Omega-3-mediated improvement of postischemic contractile recovery independent of vascular function (39) has been attributed at least in part to the incorporation of these PUFA into membrane phospholipids. Systemic estrogen has also been shown to afford protection against arrhythmogenesis, ischemia-reperfusion injury, and myocardial hypertrophy (22, 35, 43). Various in vitro studies suggest that the biological actions and cellular
targets of omega-3 PUFA and estrogen may be similar, suggesting that omega-3 PUFA may exert a greater effect in females after a period of estrogen withdrawal; albeit a possible convergence of action has not been directly investigated experimentally (7, 18, 27, 33, 47).

Given the lack of available female-specific experimental data, and in the absence of convincing clinical findings relating to the cardiovascular efficacy of omega-3 PUFA in women, a case for omega-3 cardioprotection in females has not been clearly established. Therefore, the aim of this study was to determine whether dietary omega-3 PUFA supplementation could provide cardioprotection in female mice, specifically by enhancing the recovery of ex vivo hearts to an ischemic insult. Cardiac hypertrophy is associated with increased risk of arrhythmia and vulnerability to ischemia and reperfusion injury (14, 24) and hence is a pathological condition that would be expected to be favorably modulated by the protective actions of omega-3 PUFA supplementation. Thus our study included a cohort of female mice additionally compromised by an underlying genetic predisposition for cardiac hypertrophy. Our experiments utilized the angiotensinogen-overexpressing mouse, a model of load-independent cardiac hypertrophy resulting from amplified cardiac-specific angiotensin II (Ang II) production (30). We hypothesized that dietary FO supplementation would be most efficacious (in enhancing postischemic recovery and suppressing arrhythmias) in females where there is an underlying predisposition for cardiac hypertrophy and additionally that a protective effect of dietary FO would be more prominent in the context of systemic estrogen depletion.

MATERIALS AND METHODS

The project was approved by the University of Melbourne Animal Ethics Committee (AEC Project 05173).

Experimental model. All animals were caged in the manner specified by the Prevention of Cruelty to Animals Act 1986 and NHMRC/CSIRO/ACC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997). The generation and characterization of a transgenic heterozygous mouse (TG1306/IR) harboring multiple copies of a transgene expressing the rat angiotensinogen gene under the α-myosin heavy chain promoter have been reported previously (9, 30). A colony of these mice was established in the Biological Research Facility (Department of Physiology, University of Melbourne). In this study, female age-matched transgenic mice (TG) and littermate wild-type (WT) controls were investigated with the use of a 4-wk treatment (the reported time required to achieve maximum membrane omega-3 incorporation) (37) terminating at age 34 wk. At the conclusion of the 4-wk treatment period, mice were anesthetized with pentobarbital sodium (100 mg/kg), and hearts were excised and perfused ex vivo (described below). In a separate cohort of mice, hearts were excised, adipose tissue and lungs removed, and hearts blotted dry and weighed. Atria and ventricle were then separated and weighed. Tissues were snap frozen in liquid and stored at −80°C until determination of membrane fatty acid composition.

Diets. During the treatment period, mice were fed ad libitum a fully fabricated phytoestrogen-free diet (based on the American Institute of Nutrition standard diet, 1993; AIN193), prepared at the Smart Foods Center, University of Wollongong (P. L. McLennan). The control diet (CTR) was specified to be representative of a “standard” commercial chow diet with minimum nutritional level omega-3 content and was fabricated (% of dry weight) from 50.5% cornstarch, 10% sucrose, 15% casein, 5% gelatin, 5% fiber, 3.5% mineral mix, 1% vitamin mix, and 10% oil (50% sunflower seed oil and 5% olive oil). The FO omega-3-rich diet comprised 10% oil content consisting of 7% tuna fish oil (Nu-Mega Ingredients, Brisbane, QLD, Australia) and 3% olive oil (FO diet). The FO diet was matched for total level of fatty acid polyunsaturation and caloric content to the CTR diet (1).

Cardiac membrane phospholipid fatty acid analysis. Total nonfractionated ventricular membrane phospholipid fatty acids were extracted from 70–100 mg of ventricular tissue using a modification of the Folch method as previously described (31). Phospholipids were isolated by solid-phase extraction, using silica Sep-pak cartridges.

Figure 1. Body weight and cardiac growth of female angiotensinogen transgenic mice (TG, hatched bars) at diet treatment completion (4 wk), age 34 wk, compared with littermate controls [wild-type (WT), open bars]. Data are presented as means ± SE (n = 8/group). Two-way ANOVA (factors, genetic type, and diet). A: body weight: WT vs. TG, *P < 0.005; control diet (CTR) vs. fish oil diet (FO), #P = 0.483; interaction factor P = 0.507. B: heart weight: WT vs. TG, *P < 0.001; CTR vs. FO, *P = 0.081; interaction factor P = 0.272. C: cardiac weight index: WT vs. TG, *P = 0.014; heart weight normalized to body weight (mg/g): WT vs. TG, *P = 0.001; CTR vs. FO, #P = 0.014; interaction factor, P = 0.422.
Cardiac oxygen consumption (MV\textsubscript{O2}) was determined by measuring the coronary flow rate and the partial pressure of oxygen (P\textsubscript{O2}) (ABL 615; Radiometer, Brønshøj, Denmark) of both the perfusate and the coronary effluent at 5-min reperfusion. MV\textsubscript{O2} was expressed as mol O2/min per gram wet weight using the equation: MV\textsubscript{O2} = P\textsubscript{O2} (arterial-venous) × solubility of O2/mmHg × coronary flow rate/wet heart weight (39). Hearts were excluded from the study where the baseline cardiac function was unstable, as previously defined in detail (20).

Arrhythmia analysis. The extent of ectopy during the first 10 min of reperfusion was assessed from the ventricular pressure traces, and given by the equation: number of ectopic beats/total beats × 100. Arrhythmias were also classified by type according to the following designations: ventricular premature beats (VPB; early contraction before relaxation), bigeminy (a variant of a VPB: paired beats occurring in repetition), potentiated contraction (normal sinus rhythm, slight delay, subsequent increased single contraction, resumption of sinus rhythm), ventricular tachycardia (VT; 4 or more consecutive VPBs), and ventricular fibrillation (VF; barely discernable beat, developed pressure <5 mmHg), as previously described (19).

Bilateral ovarioectomy. To withdraw gonadal estrogen, mice from each experimental group (i.e., CTR WT and TG and FO WT and TG) underwent bilateral ovarioectomy or sham surgery. Mice were anesthetized with isoflurane (2.4% and 200–220 ml airflow; Univentor U400, Zejtun, Malta). A small transverse incision was made to expose the uterus, and the uterine horns were ligated just distal to the ovarian pedicle (5–0 silk; Dynek, London, UK). Ovaries were removed (OVX) and the incision closed by suture. After surgery, mice were monitored daily for 4-wk diet treatment period until euthanized for isolated heart perfusion experiments.

A period of 4 wk of estrogen withdrawal was employed on the basis of our previous findings in rats that this duration is sufficient to induce significant changes in cardiomyocyte Ca\textsuperscript{2+} kinetics (7). At the conclusion of the 4-wk intervention, uterine weight was used to confirm ovarian estrogen withdrawal. In all mice, ovarioectomy was associated with significant uterine atrophy compared with intact mice (109 ± 8 vs. 24 ± 1 mg), and uterine weights were comparable for all OVX groups.

Table 1. Membrane PUFA levels at diet completion

<table>
<thead>
<tr>
<th></th>
<th>LA</th>
<th>Arachidonic</th>
<th>EPA</th>
<th>DHA</th>
<th>Omega-3/Omega-6</th>
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<tbody>
<tr>
<td>WT</td>
<td>18.2 ± 0.6</td>
<td>20.4 ± 0.6</td>
<td>20.5 ± 0.3</td>
<td>22.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>16.3 ± 0.5</td>
<td>16.5 ± 0.5</td>
<td>17.0 ± 0.1</td>
<td>17.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>2.9 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.763</td>
<td>0.240</td>
<td>0.292</td>
<td>0.244</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>0.001</td>
<td>0.001</td>
<td>0.010</td>
<td>0.014</td>
<td></td>
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</tbody>
</table>

Data are presented as a percentage of total membrane phospholipid fatty acids (means ± SE; n = 3–5/group). 2-way ANOVA (factors, genetic type, and diet), P, probability. Eicosapentaenoic acid (EPA) was analyzed by unpaired t-test. PUFA, polyunsaturated fatty acid; CTR, control diet; FO, fish oil; LA, linoleic acid; DHA, docosahexaenoic acid; WT, wild-type mice; TG, transgenic mice.

(l)Cardiovascular Hypertrophy in Females

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Table 2. Baseline ex vivo functional parameters

<table>
<thead>
<tr>
<th></th>
<th>LVDevP, mmHg</th>
<th>+dP/dt, mmHg/s</th>
<th>−dP/dt, mmHg/s</th>
<th>HR, beats/min</th>
<th>Flow, ml·min\textsuperscript{-1}·g\textsuperscript{-1}</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>163 ± 6</td>
<td>5089 ± 537</td>
<td>−3689 ± 194</td>
<td>347 ± 18</td>
<td>15.6 ± 1.5</td>
</tr>
<tr>
<td>FO</td>
<td>153 ± 11</td>
<td>5554 ± 422</td>
<td>−3694 ± 164</td>
<td>348 ± 17</td>
<td>16.3 ± 1.5</td>
</tr>
<tr>
<td>CTR</td>
<td>143 ± 7</td>
<td>4871 ± 417</td>
<td>−3723 ± 255</td>
<td>371 ± 23</td>
<td>15.0 ± 1.6</td>
</tr>
<tr>
<td>TG</td>
<td>150 ± 10</td>
<td>4643 ± 365</td>
<td>−3630 ± 214</td>
<td>353 ± 22</td>
<td>16.3 ± 0.9</td>
</tr>
<tr>
<td>P</td>
<td>0.199</td>
<td>0.408</td>
<td>0.700</td>
<td>0.454</td>
<td>0.828</td>
</tr>
<tr>
<td>TG</td>
<td>0.839</td>
<td>0.706</td>
<td>0.892</td>
<td>0.672</td>
<td>0.484</td>
</tr>
<tr>
<td>P</td>
<td>0.344</td>
<td>0.553</td>
<td>0.876</td>
<td>0.639</td>
<td>0.868</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 8/ group). All parameters were measured after 20-min normoxic perfusion (equilibration). 2-way ANOVA, P, probability. LVDevP, left ventricular developed pressure; +dP/dt, rate of pressure development; −dP/dt, rate of pressure decline; HR, heart rate.
Statistical analyses. Data are expressed as means ± SE. Group sizes for cardiac weight index determinations and for functional analyses ranged from \( n = 8–12 \), and for membrane analyses, \( n = 3–4 \) (these data exhibit low variance). Data were analyzed using two-way ANOVA and with repeated measures where appropriate. Multiple-comparisons post hoc analysis was performed using Tukey HSD. Arrhythmia duration data were analyzed with a nonparametric Kruskal-Wallis test and incidence analyzed with the Fisher’s exact test. All data were considered significant at \( P < 0.05 \). Statistical calculations were performed using the SPSS V.15.0 (SPSS, Chicago, IL).

RESULTS

Somatic growth, cardiac growth, and ventricular membrane fatty acid composition. Body mass and food consumption were tracked throughout the 4-wk diet treatment period. The mean
food intake in g/mouse per day was not different across diet group (CTR diet 3.1 ± 0.3 g, FO diet 2.8 ± 0.3 g, P = 0.459). At the end of the diet treatment period, body mass was similar between all groups (Fig. 1A). Heart mass and the normalized cardiac weight index (heart mass normalized to body mass) were significantly greater for TG hearts compared with WT hearts in both dietary groups (P < 0.001, n = 8/group; Fig. 1, B and C). FO diet was associated with smaller normalized heart mass for both TG (by 10%) and WT hearts (by 5%) compared with strain-matched CTR diet-fed mice (P = 0.014, Fig. 1C) but no significant diet and genetic type interaction was detected (Fig. 1C).

Four weeks of dietary FO markedly elevated the omega-3:omega-6 PUFA ratio of both WT and TG (Table 1). This effect was primarily achieved through decreased ventricular membrane linoleic and arachidonic acid and elevated EPA and DHA. For FO-fed TG mice there was a small but selectively greater increase in DHA and in the omega-3:omega-6 PUFA ratio compared with diet-matched WT (Table 1).

Effects of dietary FO on cardiac performance pre- and postischemia. Ex vivo isovolumic mechanical function, coronary flow, and MV˙O₂ were measured pre- and postischemia in hearts of WT and TG mice (n = 8/group). Under basal conditions (normoxia), there were no significant differences between any of the groups (Table 2). Global ischemia (15 min) rapidly reduced contractile function in all of the groups, and all hearts had resumed mechanical function by the end of reperfusion (30 min), albeit at a reduced level compared with baseline (Fig. 2). The time course of postischemic recovery throughout reperfusion is shown in Fig. 2 and depicted as nonnormalized and baseline-normalized values. For the nonnormalized data, postischemic developed pressure (Fig. 2A), the RPP (Fig. 2C), rate of contraction (+dP/dt, Fig. 2E), and rate of pressure decline (−dP/dt, data not shown) were all not significantly different between WT and TG hearts or between the CTR- and FO-fed hearts. For intrinsic HR (Fig. 2G), a significant interaction between genetic type and diet (P = 0.026) was detected, indicating that the FO diet had a differential effect in WT (increased HR) and TG (lowered HR) hearts.

Normalization of postischemic function to pres ischemic “baseline” values (Fig. 2, right) did not reveal any further significant differences across any of the groups. Hearts of CTR-fed TG appeared to recover a slightly higher level of developed pressure, RPP, rate of contraction (+dP/dt) (Fig. 2, B, D, and F), and rate of pressure decline (−dP/dt, data not shown) compared with all other groups, but this effect was not significant for any parameters (interaction P = 0.394, P = 0.366, P = 0.221, P = 0.209 for each parameter, respectively). Baseline-normalized intrinsic HR followed a similar pattern to the nonnormalized data, but the interaction effect (differential effect of FO diet in TG and WT) did not quite attain statistical significance (P = 0.072, Fig. 2H).

Coronary flow and myocardial oxygen consumption were markedly reduced postischemia (compared with baseline), by ~10–30% (Fig. 3 vs. Table 2). Dietary FO did not alter coronary regulation for either WT or TG mice, compared with genetically matched CTR-fed mice (Fig. 3A). Myocardial oxygen consumption paralleled coronary flow (Fig. 3B), indicating a direct relationship between oxygen delivery and extraction for hearts of all groups.
Fig. 4. Effects of 4-wk dietary FO supplementation (compared with CTR) in ovariectomized (OVX) WT (circles) and TG (triangles) on ex vivo contractile recovery and coronary flow following 15-min global no-flow ischemia. Time course shows basal (time point B), which was measured after 20-min equilibration and time points at 5-min intervals during 30-min reperfusion. Data are presented as means \( \pm \) SE (\( n = 8–12 \)/group). Two-way ANOVA (factors, genetic type, and diet) with repeated measures. A: RPP (developed pressure \( \times \) heart rate); WT vs. TG, \( *P = 0.016 \); CTR vs. FO, \( P = 0.335 \); interaction factor, \( P = 0.615 \). B: baseline normalized rate pressure product; WT vs. TG, \( *P = 0.014 \); CTR vs. FO, \( P = 0.175 \); interaction factor, \( P = 0.873 \). C: rate of contraction \( (+dP/dt) \); WT vs. TG, \( *P = 0.005 \); CTR vs. FO, \( P = 0.341 \); interaction factor, \( P = 0.417 \). D: baseline normalized rate pressure product; WT vs. TG, \( *P = 0.003 \); CTR vs. FO, \( P = 0.207 \); interaction, \( P = 0.813 \).

with hearts of OVX WT (CTR 13.8 \( \pm \) 1.1 vs. 16.5 \( \pm \) 1.0 ml\( \cdot \)min\(^{-1} \cdot \)g\(^{-1} \) wet wt, \( P = 0.009 \); 7.4 \( \pm \) 0.5 vs. 8.8 \( \pm \) 0.4 \( \mu \)mol O\(_2\)\( \cdot \)min\(^{-1} \cdot \)g\(^{-1} \) wet wt, \( P = 0.006 \)).

The time course of postischemic recovery throughout reperfusion (of OVX hearts) is shown in Fig. 4 and depicted as nonnormalized and baseline-normalized values. Overall throughout reperfusion, OVX TG hearts (from both diet groups) exhibited enhanced recovery of RPP product and rate of pressure development \( (+dP/dt) \), by \( \sim 25\% \) (Fig. 4, A and C), compared with OVX WT hearts. Dietary FO did not enhance mechanical recovery of OVX WT or TG hearts for any of the parameters measured, including RPP (Fig. 4A), rate of pressure development \( (+dP/dt) \), and developed pressure or rate of pressure decline \( (−dP/dt) \) (data not shown). In the baseline-normalized data (Fig. 4, right), a similar pattern to the nonnormalized values was observed. In contrast to the ovary-intact mice (Fig. 2), FO diet did not significantly alter intrinsic HR of WT or TG hearts. Similar to the hearts of ovary-intact mice (Fig. 3), dietary FO did not significantly alter coronary regulation for either WT or TG mice, compared with genetically matched CTR-fed mice (Fig. 5A). Myocardial oxygen consumption paralleled coronary flow (Fig. 5B).

Interestingly, in general, ovariectomy was associated with an apparent increase in the propensity for mechanical alternans (beat-to-beat alterations in twitch amplitude \( \geq 5 \) mmHg at a constant HR) (19). Thirty-one percent of hearts \( (n = 3/141) \) from the OVX groups displayed alternans compared with only 6\% of hearts \( (n = 2/32) \) from the ovary-intact groups, and notably this vulnerability was not detected in all in the ovary-intact WT groups \( (P = 0.001) \). Furthermore, separation of the WT hearts by diet groups revealed that the incidence of alternans in CTR WT was markedly greater in the OVX hearts \( (n = 6/10) \) than in the ovary-intact group, where there was no occurrence \( (n = 0/8, P = 0.01) \). This difference was not significant in the FO-fed WT OVX (OVX \( n = 3/8 \) vs. intact \( n = 0/8) \).

**Dietary FO supplementation and postischemic arrhythmia.** Arrhythmia incidence was evaluated during the first 10 min of reperfusion from the ventricular pressure record and classified as previously described (19). In the ovary-intact and OVX groups, no significant differences in the incidence of VPB, VT (4 or more consecutive premature beats), potentiated contraction, or the duration of bigeminy and VF were detected between WT and TG hearts. Hearts of FO-fed mice did not exhibit an altered incidence of these arrhythmia types in either the ovary-intact or OVX groups (Table 3).

To obtain an aggregated “snap shot” of arrhythmia incidence, the percentage ectopy was determined by expressing the number of arrhythmic beats as a percentage of total beats. Overall (i.e., from both diet groups), TG hearts of both ovary-intact (Fig. 5A) and OVX groups (Fig. 5B) exhibited a smaller percentage ectopy compared with WT hearts. Compared with mice fed the CTR diet, dietary FO had no effect on the extent of ectopy exhibited in the reperfused hearts in both ovary-intact (Fig. 6A) and OVX groups (Fig. 6B).

**DISCUSSION**

This is the first experimental study to address the question of whether dietary omega-3 PUFA intervention confers cardioprotection in the ischemic female heart. The major findings of this study are that, in the female murine heart, dietary FO 1) modestly suppresses hypertrophic growth 2) does not enhance postischemic recovery in the normal or the hypertrophic heart, 3) does not enhance postischemic recovery in the normal or hypertrophic hearts of estrogen-depleted mice, and 4) does not lower the incidence of reperfusion arrhythmia in the normal or hypertrophic heart. Thus contrary to our hypothesis, we find...
Dietary FO modulates myocardial membrane composition and growth. The finding that dietary FO suppresses heart growth is important. FO-fed TG hearts had a greater proportion of membrane-incorporated omega-3 DHA (22:6 omega-3) compared with WT hearts, which may be linked to hypertrophic growth suppression. There have been only two other (published) experimental studies that have investigated the effects of dietary omega-3 PUFA on cardiac hypertrophic growth, and these studies have been in male rodents. Duda et al. (11) reported that, in a rat model of pressure overload, the increase in left ventricular mass was less marked in the FO-fed group compared with rats fed the contrast diet (rich in saturated fat). A FO diet-induced reduction in ventricular mass in a murine model of systemic carnitine deficiency (JVS mice) where cardiac enlargement occurs secondary to lipotoxicity has also been reported (45). The mechanisms leading to regression of cardiac hypertrophy in vivo remain to be elucidated. Takahashi et al. (45) observed that dietary FO prevented the membrane translocation of several cytosolic protein kinase C isoforms and suggested that this may be the mechanism for the hypertrophy suppression in the JVS mice. In vitro studies in isolated neonatal rat cardiac myocytes (sex of rats not reported) have demonstrated that DHA and EPA inhibit the growth response (i.e., protein synthesis) induced by phenylephrine and endothelin-1 stimulation via the Ras/Raf/Erk1/2 and JNK signaling pathways (41, 42). In the present study, the growth reduction effect of FO diet was observed without a prior hemodynamic loading stimulus and therefore likely reflects a direct modulation of intrinsic myocardial trophic maintenance signaling. It has been proposed that in females adaptive hypertrophic growth plays a more important role in systolic functional maintenance in compromised situations (36). A detailed molecular understanding is required to discern whether growth suppression associated with omega-3 intervention in females is cardioprotective.

Postischemic recovery in TG females is not undermined. The hypertrophic TG female hearts did not exhibit contractile dysfunction under basal conditions (when compared with WT), no evidence of a protective effect of dietary FO on myocardial postischemic recovery or arrhythmia suppression in females in the normal heart, the hypertrophic heart, or the estrogen-withdrawn heart. An important finding of this study is that sex should be considered in future investigations of the cardiac mechanisms of action of omega-3 PUFA.

### Table 3. Type and incidence of reperfusion arrhythmias in hearts of CTR-fed and FO-fed intact and ovariectomized WT and TG mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>FO</th>
<th>WT</th>
<th>FO</th>
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<tbody>
<tr>
<td>Intact</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPB (incidence)</td>
<td>104±16 (8)</td>
<td>66±14 (8)</td>
<td>77±29 (8)</td>
<td>62±18 (8)</td>
</tr>
<tr>
<td>VT (incidence)</td>
<td>2±1 (3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>potentiated (incidence)</td>
<td>5±2 (8)</td>
<td>18±5 (7)</td>
<td>39±8 (7)</td>
<td>46±25 (6)</td>
</tr>
<tr>
<td>bigeminy (seconds)</td>
<td>82±21 (8)</td>
<td>61±22 (6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>VF (seconds)</td>
<td>51 (1)</td>
<td>108 (1)</td>
<td>ND</td>
<td>ND</td>
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<td>Ovariectomized</td>
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<tr>
<td>VPB (incidence)</td>
<td>70±9 (10)</td>
<td>89±24 (8)</td>
<td>62±11 (12)</td>
<td>67±13 (11)</td>
</tr>
<tr>
<td>VT (incidence)</td>
<td>2±1 (3)</td>
<td>9 (1)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>potentiated (incidence)</td>
<td>25±11 (6)</td>
<td>20±8 (6)</td>
<td>24±16 (7)</td>
<td>21±13 (6)</td>
</tr>
<tr>
<td>bigeminy (seconds)</td>
<td>56±16 (8)</td>
<td>36±16 (5)</td>
<td>55±17 (8)</td>
<td>55±15 (7)</td>
</tr>
<tr>
<td>VF (seconds)</td>
<td>344±120 (2)</td>
<td>174 (1)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Assessment of ex vivo arrhythmia during the first 10 min of reperfusion. Arrhythmias were identified from the ventricular pressure record, using the criteria described in MATERIALS AND METHODS. Numbers in parentheses indicate the number of hearts exhibiting the type of arrhythmia. SE is omitted where this number is 1 only. Ventricular premature beat (VPB), ventricular tachycardia (VT), and potentiated contraction are expressed as number of events (incidence) occurring during the first 10 min of reperfusion and shown as means ± SE for the number of hearts in parentheses. Bigeminy and ventricular fibrillation (VF) are presented as duration (seconds), occurring during the first 10 min of reperfusion and shown as means ± SE for the number of hearts in parentheses. No significant differences were detected. ND, not detected.
which contrasts with our previous observations in male TG of similar age (20). Although our earlier study did not involve a specified diet and comparison, the diet used most closely resembles the CTR diet described here. The present finding accords with other reports in the literature that have shown that the occurrence, onset, and progression of cardiac hypertrophy are frequently different in females compared with males (10). In addition, this study found that the TG female hearts exhibit an intrinsic functional resistance to ischemia and reperfusion (equivalent to WT). Hypertrophic exacerbation of ischemia and reperfusion damage has been previously observed in some experimental models where cardiac growth and function are modified by systemic loading conditions. This study shows that in the female, under these experimental conditions, primary chronic hypertrophy occurring without hemodynamic complication does not impair postischemic mechanical recovery or promote posts ischemic arrhythmia. This finding is consistent with previous studies in the rat that have shown that female hearts are protected from ischemia-reperfusion, exhibiting lower incidence of arrhythmia, improved recovery of contractility, and decreased necrosis when compared with males (2, 22, 35). These differences are largely attributed to sex steroids although the cellular and molecular mechanisms underlying sex differences in cardiac function remain unresolved (25). The sarcolemmal ATP-sensitive potassium channel may play a role because expression of this channel is estrogen modulated and pharmacological block has been shown to abolish sex differences in ischemia-reperfusion damage in ex vivo hearts (23).

**FO may not confer protection in the female heart.** Contrary to our primary hypothesis, the extent of postischemic mechanical recovery was found not to be enhanced in the hearts of FO-fed TG (or WT), when compared with CTR-fed mice. Dietary FO, which is generally considered to be cardioprotective (29, 31, 39), albeit not always (4), had no effect on postischemic mechanical recovery or arrhythmia suppression (VPB, VT, VF) in WT hearts. In younger animals fed diets identical to those used in the present study, we have previously identified a postischemic protective effect of FO in WT and TG males (21). In the present study, hearts of FO-fed TG hearts did exhibit a lower intrinsic HR during reperfusion compared with TG CTR-fed mice. This finding is consistent with previous studies in the rat and human (1) and has been suggested to be beneficial since a lower resting HR reduces risk of arrhythmia. However, surprisingly in this study we did not find an arrhythmic effect of dietary FO on postischemia arrhythmia incidence in the TG (or WT) hearts when compared with CTR-fed TG.

Review of the literature provides a basis for inferring that omega-3 may act at similar cellular targets to estrogen (7, 18, 27, 33, 47). Dietary omega-3 PUFA and estrogen have been both observed to modulate cardiomyocyte ion homeostasis, particularly the ionic fluxes involved in excitation-contraction coupling, and have been linked with intracellular Ca(2+)-dependent downstream signaling. In addition, omega-3 PUFA and estrogen may confer benefit by directly targeting the genome (i.e., bind to nuclear transcription factors). In the estrogen-replete female, activation and transcription of genes involved with cardiac remodeling, cellular repair, and mitochondrial biogenesis may already be invoked (by estrogen), hence precluding the potential for further omega-3 PUFA action. Thus we advanced a second hypothesis that the effects of dietary FO may be masked in the WT and TG hearts attributable to the already “protected” state of the estrogen-replete female.

**Withdrawal of ovarian sex hormones does not unmask a cardioprotective action of dietary FO.** To evaluate whether systemic estrogen was “preempting” a protective effect of omega-3 PUFA (i.e., the effects of both estrogen and omega-3 PUFA are not additive), we compared the responses of OVX WT and TG mice to FO dietary intervention. It has been previously established that a 4-wk period of experimental estrogen depletion by ovariectomy is sufficient to reverse the functional cardiomyocyte modeling conferred by endogenous estrogen (7). OVX CTR-fed WT hearts exhibited a distinct vulnerability to ischemia, displaying a markedly higher incidence of mechanical alternans suggestive of abnormal calcium handling (5, 12). Data subanalysis suggests that FO may selectively influence alternans occurrence in WT-OVX. Although mechanical alternans occurrence cannot be strictly considered of arrhythmogenic origin without additional characterization, this observation does provide a possible avenue for further cellular investigations. Previous dietary and acute (in vitro) studies (in males) have indicated that omega-3 PUFA confers arrhythmia protection through favorably modulating intracellular calcium flux (18, 32, 47). Our finding was that dietary FO did not enhance postischemic mechanical recovery in the estrogen-depleted WT or TG hearts. Moreover, there was no beneficial effect of dietary FO on the incidence of reperfusion arrhythmia.

**Fig. 6.** Assessment of ex vivo arrhythmia incidence during the first 10 min of reperfusion in WT and TG mice 4 wk after dietary FO supplementation or CTR. Data (means ± SE; n = 8–12/group) are expressed as a percentage ectopy (aryhythmic beats/total beats × 100). ANOVA (factors, genetic type, and diet): A: hearts of ovary-intact WT and TG mice: WT vs. TG, *P = 0.030; CTR vs. FO, P = 0.299; interaction factor, P = 0.947. B: hearts of OVX WT and TG mice: WT vs. TG, *P = 0.015; CTR vs. FO, P = 0.529; interaction factor, P = 0.245.
These findings, in particular the lack of effect on reperfusion arrhythmias, represent intriguing experimental outcomes given the consistent (but not universal) reports in the literature that dietary FO-derived omega-3 PUFA are antiarrhythmic in male rodents following ischemia and reperfusion (29, 31, 39). This unanticipated finding adds to the emerging evidence that omega-3 PUFA may not be antiarrhythmic in all contexts. Given that the diets fabricated for this study were matched for total PUFA content, these findings may suggest that, in females, both omega-3 and omega-6 PUFA classes exert similar effects. The absence of a cardioprotective FO effect in the estrogen-deplete female in this study prompts consideration of the proposition that testosterone is important in males in mediating the actions of omega-3 intervention. There is evidence to suggest that testosterone may reduce the activities of the enzymes involved in the desaturation process of the 18-carbon chain PUFA to the longer more unsaturated PUFA (28). Thus supplementation studies with long-chain omega-3 PUFA (e.g., DHA and EPA) may not achieve the same outcome in females because of relatively greater intrinsic capacity to produce these PUFA endogenously.

An additional finding of this study was that postischemia differences in the extent of mechanical recovery were evident in O VX TG (compared with intact) that were not apparent in the O VX WT (Fig. 4C vs. Fig. 2F). In these TG hearts, ovariectomy was associated with a higher level of intrinsic contractility (i.e., +dP/dt) postischemia compared with genotype-matched intact mice. Thus our findings suggest that the absence of circulating sex hormones impacts differently on the myocardium when there is a chronic local activation of RAS. Previous studies have reported an Ang II-estrogen interaction (38, 48). Chronic infusion with Ang II was associated with a greater pressor effect in O VX females and males compared with intact females, indicating a protective role of ovarian hormones against Ang II-induced hypertension (48). The finding that postischemic excitation-contraction coupling is altered suggests that in vivo the local cardiac growth mediator milieu may interact with systemic estrogen to modulate excitation-contraction coupling.

Previous studies have shown that Ang II stimulates increases in myocyte cytosolic free Ca\(^{2+}\) by activating voltage-sensitive Ca\(^{2+}\) channels, producing a subsequent increase in contractility (13). Ang II modulates beat-to-beat Ca\(^{2+}\) involved in excitation-contraction coupling, and an Ang II-mediated positive inotropic effect on isolated rat myocytes has been demonstrated (8). The positive inotropic effects of Ang II have been attributed to an increase in the Ca\(^{2+}\) transient with (15) or without (46) an increase in myofilament sensitivity to Ca\(^{2+}\). In contrast, it has been shown that estrogen suppresses cellular Ca\(^{2+}\) flux (6, 7, 33). Thus, in the TG, the removal of ovarian estrogen would be expected to enhance intracellular Ca\(^{2+}\) flux, and, in a relatively low workload environment (as in this study, with a modest ischemic insult), an augmented Ca\(^{2+}\) flux may improve inotropic state—confering relative “hypercontractility.” However, more severe in vivo ischemic or workload demands could be expected to unmask mechanical deficits associated with higher resting intracellular Ca\(^{2+}\). In such circumstances, it might be predicted that Ang II hypercontractility postischemia undermines long-term mechanical stability and recovery. Interestingly, in a different pathological setting of a disease model of familial hypertrophic cardiomyopathy, dietary phytoestrogen enrichment (soy intake) has been shown to exacerbate mechanical dysfunction (44). In this context, it may be that high levels of ingested exogenous estrogens undermine the supply of activator Ca\(^{2+}\) for excitation-contraction coupling and impair the adaptive response to counter genetic abnormality. Further work is required to understand the effects of exogenous and endogenous estrogens on Ca\(^{2+}\) modulation in hypertrophic disease states. The importance of using fully specified diets rather than diets of regionally and seasonally variable phytoestrogen content is also increasingly apparent.

In summary, this study provides the first experimental evaluation of the effects of dietary FO in modulating the myocardial responses of female hearts to ischemic challenge. Our findings indicate a lack of selective cardioprotective effect of FO dietary intervention determined by assessment of mechanical and arrhythmic activity in the murine ex vivo heart model. Furthermore, relative to the CTR diet, FO intervention was without functional benefit even in the presence of cardiac hypertrophy (induced by cardiac Ang II overproduction) or in the context of chronic systemic estrogen withdrawal (surgically simulated “menopause”). Interestingly, FO diet exerted a significant (modest) cardiac growth suppression effect, which was not selective for hypertrophic hearts. These novel experimental findings highlight the importance of gaining a more complete understanding of the role of omega-3 PUFA in regulating growth and function specifically in the female heart. The assumed benefits of omega-3 intervention/supplementation which have been largely identified in male cohort studies may not necessarily translate to females.

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