Treatment with Docosahexaenoic Acid, but Not Eicosapentaenoic Acid, Delays Ca^{2+} -Induced Mitochondria Permeability Transition in Normal and Hypertrophied Myocardium

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ABSTRACT

Intake of fish oil containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) prevents heart failure; however, the mechanisms are unclear. Mitochondrial permeability transition pore (MPTP) opening contributes to myocardial pathology in cardiac hypertrophy and heart failure, and treatment with DHA + EPA delays MPTP opening. Here, we assessed: 1) whether supplementation with both DHA and EPA is needed for optimal prevention of MPTP opening, and 2) whether this benefit occurs in hypertrophied myocardium. Rats with either normal myocardium or cardiac hypertrophy induced by 8 weeks of abdominal aortic banding were fed one of four diets: control diet without DHA or EPA or diets enriched with either DHA, EPA, or DHA + EPA (1:1 ratio) at 2.5% of energy intake for 17 weeks. Aortic banding caused a 27% increase in left ventricular mass and 25% depletion in

DHA in mitochondrial phospholipids in rats fed the control diet. DHA supplementation raised DHA in phospholipids ~2-fold in both normal and hypertrophied hearts and increased EPA. DHA + EPA supplementation also increased DHA, but to a lesser extent than DHA alone. EPA supplementation increased EPA, but did not affect DHA compared with the control diet. Ca^{2+} -induced MPTP opening was delayed by DHA and DHA + EPA supplementation in both normal and hypertrophied hearts, but EPA had no effect on MPTP opening. These results show that supplementation with DHA alone effectively increases both DHA and EPA in cardiac mitochondrial phospholipids and delays MPTP and suggest that treatment with DHA + EPA offers no advantage over DHA alone.

Introduction

Cardiac hypertrophy in response to sustained elevation in blood pressure is a strong, independent risk factor for heart failure (Levy et al., 1990). Intake of ω -3 polyunsaturated fatty acids (ω -3 PUFA) from fish, specifically docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3), is associated with lower risk of development of heart failure, and treatment of patients with established heart failure with DHA + EPA decreases death and hospi-

talization (Mozaffarian et al., 2005; GISSI-Hf Investigators, 2008; Duda et al., 2009a). We have found that dietary supplementation with DHA + EPA prevents expansion of the chamber volume of the left ventricle (LV) and myocardial pathology in rats subjected to chronic arterial pressure overload induced by aortic constriction (Duda et al., 2007, 2009b; Shah et al., 2009). The mechanisms responsible for these effects are complex and unclear, but may be partially attributable to improved function of cardiac mitochondria.

Optimal mitochondrial function is critical for maintaining myocardial ATP concentration and preventing apoptosis, making this organelle's integrity and function crucial for cardiomyocyte survival (Stanley et al., 2005; Neubauer, 2007). Recent studies suggest that increased vulnerability to opening of the mitochondrial permeability transition pore

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ABBREVIATIONS: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; MPTP, mitochondrial permeability transition; PUFA, polyunsaturated fatty acids; CTRL, control; AAB, abdominal aortic banding; LV, left ventricle; MHC, myosin heavy chain; ANF, atrial natriuretic factor; BSA, bovine serum albumin; SSM, subsarcolemmal mitochondria; IFM, interfibrillar mitochondria; MOPS, 4-morpholinepropanesulfonic acid; RCR, respiratory control ratio.

(MPTP) can accelerate the development of heart failure. The MPTP is a large-diameter (3 nm), high-conductance, voltage-dependent channel that allows passage of water, ions, and molecules up to ~1.5 kDa (Halestrap, 2009). Formation of the MPTP in cardiac mitochondria is associated with cardiomyocyte death, tissue injury, and poor recovery from ischemia/reperfusion, and inhibition of MPTP is a putative therapeutic target for the prevention and treatment of heart failure (Javadov and Karmazyn, 2007; Baines, 2009; Halestrap, 2009).

Although the molecular components and structure of the pore are unclear, our evidence suggests that it depends on the phospholipid composition of mitochondrial membranes (O'Shea et al., 2009). We found that supplementation with a mixture of DHA + EPA that is similar to widely used dietary fish oil supplements delayed Ca^{2+} induced MPTP opening (O'Shea et al., 2009). These changes were accompanied by an increase in DHA and EPA in mitochondrial phospholipids and a decrease in arachidonic acid (ARA; 20:4n-6). ARA is an ω -6 PUFA that has been implicated in the induction of MPTP (Williams and Gottlieb, 2002; Penzo et al., 2004; Kinsey et al., 2007). We have compared the independent effects of dietary supplementation with DHA versus EPA and found that supplementation with DHA alone elevated both DHA and EPA and decreased ARA in mitochondrial phospholipids and delayed Ca^{2+} -induced MPTP opening (Khairallah et al., 2010). On the other hand, EPA supplementation increased EPA, but failed to increase DHA or delay MPTP opening. Direct comparison of DHA supplementation to the typically used DHA + EPA mixtures has not been reported.

Cardiac hypertrophy can result in mitochondrial dysfunction, cardiomyocyte apoptosis, and heart failure (Kang et al., 2004; Bugger et al., 2010). Cardiac mitochondria from rats with volume overload-induced cardiac hypertrophy are more susceptible to MPTP opening (Marcil et al., 2006). This effect may be caused by depletion of DHA from mitochondrial membrane phospholipids, thus dietary supplementation with DHA or DHA + EPA should restore membrane DHA, improve mitochondrial function, and prevent MPTP opening. Because EPA is not readily elongated to DHA in heart tissue, EPA would not exert this beneficial effect. Therefore, the goal of the present study was to compare the effects of DHA supplementation with DHA + EPA and EPA alone in normal and hypertrophied myocardium. We hypothesized that dietary supplementation with DHA alone is superior to EPA or DHA + EPA in increasing mitochondrial DHA, decreasing ARA, and delaying Ca^{2+} -induced MPTP. Rats were subjected to either abdominal aortic banding or sham surgery to establish pressure overload-induced hypertrophy and were treated for 17 weeks with the control (CTRL) diet without DHA or EPA or diets enriched with either DHA, EPA, or DHA + EPA (1:1 ratio). Myocardial structure and function were assessed by echocardiography, and mitochondria were isolated for evaluation of phospholipid fatty acid composition and Ca^{2+} -induced MPTP opening.

Materials and Methods

Experimental Design. The animal protocol was approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee and conducted according to the *Guidelines for the Care and Use of Laboratory Animals* (National Institutes of

Health publication 85-23). Investigators were blinded to treatment when measurements were performed. The animals were maintained on a reverse 12-h light/dark cycle, and all procedures were performed between 3 and 6 h from the start of the dark phase.

Male Wistar rats (Harlan, Indianapolis, IN) (180–190 g) underwent sham surgery or abdominal aortic banding and were fed a custom-manufactured purified CTRL diet that was free of DHA and EPA. The surgical procedure has been described previously in detail (Duda et al., 2007, 2009b; Shah et al., 2009). In brief, rats were anesthetized with isoflurane, the abdominal aorta was exposed by a midline incision, and a silk suture was tied against a 21-gauge needle around the abdominal aorta superior to the renal arteries. The needle was removed, leaving the aorta constricted. In sham animals the suture was left loose around the aorta.

Eight weeks after surgery LV chamber size and wall thickness were assessed by echocardiography, and animals were assigned to one of four diets: CTRL ($n = 8$ sham and 14 banded) or a diet similar to the control diet but supplemented with DHA ($n = 9$ sham and 14 banded), EPA ($n = 9$ sham and 15 banded), or DHA + EPA ($n = 8$ sham and 14 banded). Rats were assigned to treatment groups to match body mass and end systolic and diastolic diameters among groups. Echocardiography was performed again at 24 weeks after surgery. At 25 weeks after surgery (after 17 weeks of treatment) the animals were anesthetized with 2.0% isoflurane, plasma and serum were collected, and organs were immediately harvested, weighed, frozen in liquid nitrogen, and stored at -80°C for later analysis. Mitochondria were freshly isolated from the LV.

Diets. All chows were custom-manufactured (Research Diets Inc., New Brunswick, NJ), and had 68% of total energy from carbohydrate (38% of total energy from cornstarch, 5% from maltodextrin, and 25% from sucrose), 20% protein (casein supplemented with L-cystine), and 12% energy from fat. In the CTRL diet the fat was made up of 35.3% cocoa butter, 39.8% lard, 16.6% soybean oil, and 8.3% palm kernel oil (see Table 1 for fatty acid composition of the diets). The DHA diet contained 5.8% of total energy from algal oil (DHASCO; Martek Inc., Columbia, MD) that was comprised of 45.6% DHA by mass with the balance from cocoa butter and soybean oil. The EPA diet had 2.6% of energy from purified fish oil comprised of 95.5% EPA by mass (KD Pharma, Bexbach, Germany), with the balance from cocoa butter, soybean oil, safflower oil, and palm kernel oil. The DHA + EPA contained 2.87% of total energy from the high-DHA algal oil and 1.3% of the high EPA fish oil with the balance from cocoa butter, soybean oil, safflower oil, and palm kernel oil.

Echocardiography. LV size was assessed in anesthetized rats by using a high-resolution small animal imaging system (model Vevo 770, with transducer model RMV 716; VisualSonics Inc., Toronto, Canada), as described previously in detail (Duda et al., 2009b; Shah et al., 2009). In brief, rats were anesthetized with isoflurane by mask and placed supine on a heated platform, and two-dimensional cine loops and guided M-mode frames were acquired from the short and long axis. Measurements were made off-line by using software resident on the system.

TABLE 1

Fatty acid compositions of the diet expressed as the molar percent of total fatty acids in the chow

All chows had 12% of total energy from fat, 20% from protein, and 68% from carbohydrate.

Fatty Acid	Control	DHA	EPA	DHA + EPA
C12:0	3.9	2.2	3.9	2.8
C14:0	1.7	4.9	1.3	2.8
C16:0	21.6	14.7	13.1	13.8
C16:1	1.7	1.8	—	0.9
C18:0	18.9	13.8	14.0	13.9
C18:1n-9	34.9	29.7	31.6	30.6
C18:2n-6	13.9	11.1	13.0	12.1
C18:3n-3	1.8	1.3	1.3	1.3
C20:5n-3	—	—	19.1	9.6
C22:6n-3	—	19.1	—	9.6

Mitochondrial Preparation. Mitochondria were isolated as described previously (O'Shea et al., 2009; Khairallah et al., 2010). In brief, LV tissue (~500 mg) was minced and homogenized in 1:10 cold modified Chappel-Perry buffer [100 mM KCl, 50 mM MOPS, 5 mM MgSO_4 , 1 mM EGTA, 1 mM ATP, 0.2 mg/ml bovine serum albumin (BSA)], and the homogenates were centrifuged at 500g. Subsequent centrifugation allowed for separation and purification of the subsarcolemmal mitochondria (SSM). Separation of the interfibrillar mitochondria (IFM) was achieved by incubation on ice with trypsin (5 mg/g wet mass) for 10 min and subsequent centrifugations for purification. The concentration of mitochondrial protein was measured by the Lowry method using BSA as a standard.

Mitochondrial Respiration. Mitochondrial oxygen consumption was assessed in SSM and IFM as described previously in detail (O'Shea et al., 2009; Khairallah et al., 2010). Isolated mitochondria (0.5 mg mitochondrial protein/ml) were respired in buffer containing 100 mM KCl, 50 mM MOPS, 5 mM KH_2PO_4 , 1 mM EGTA, and 1 mg/ml BSA. State 3 and 4 respiration was measured by using pyruvate + malate (10 and 5 mM, respectively), palmitoylcarnitine + malate (10 and 5 mM, respectively), and succinate + rotenone (10 mM and 7.5 μM , respectively). State 4 respiration was measured with the addition of oligomycin (4 μM).

MPTP Opening. Ca^{2+} -induced MPTP opening was assessed in isolated SSM and IFM as described previously in detail (O'Shea et al., 2009; Khairallah et al., 2010). In brief, 0.5 mg of mitochondrial protein was added to Ca^{2+} and BSA-free buffer containing 5 μM EGTA, 1 mM MgCl_2 , 10 mM glutamate, and 5 mM malate. A 5 mM Ca^{2+} solution was continuously infused (1 $\mu\text{l}/\text{min}$), and extramitochondrial free Ca^{2+} was measured by using 0.1 mM Fura-6-F at 37°C with excitation wavelengths for the free and Ca^{2+} -bound forms of 340 and 380 nm, respectively, and emission wavelength of 550 nm. The cumulative Ca^{2+} load that was required to trigger the opening of the MPTP was determined from semilog plots of the extramitochondrial Ca^{2+} concentration plotted as a function of the cumulative Ca^{2+} load and was defined as the point where there was a sharp increase in extramitochondrial Ca^{2+} concentration. Analysis was done by a single investigator blinded to treatment.

Metabolic and Biochemical Parameters. Plasma-free fatty acids and triglycerides were measured by spectrophotometric enzymatic assays as described previously (Duda et al., 2009b; Shah et al., 2009). The maximal activities of isocitrate dehydrogenase, medium chain acyl-CoA dehydrogenase, and citrate synthase were measured spectrophotometrically in frozen LV tissue as described previously (Chess et al., 2008; Duda et al., 2009b; Shah et al., 2009).

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction. RNA extraction and quantitative reverse transcription-polymerase chain reaction were performed on frozen LV tissue as described previously (Duda et al., 2009b; Shah et al., 2009). Specific quantitative assays were obtained from Applied Biosystems (Foster City, CA) for ANF, $\text{MHC}\alpha$, and $\text{MHC}\beta$. RNA concentration was normalized to cyclophilin A in each sample.

Statistical Analyses. Mean values are presented $\pm$ S.E.M., and the level of significance was set at $p < 0.05$. Assessment of the effects of diet and aortic banding were made by two-way analysis of variance. The effect of diet on the concentration of extramitochondrial Ca^{2+} was evaluated with two-way analysis of variance for repeated measures within the sham and abdominal aortic banding (AAB) groups. Post hoc comparisons were made with Fisher's least significant difference test. In the animals harvested at 8 weeks differences between sham and AAB groups were assessed by Student's t test.

Results

Cardiac Mass, Function, and mRNA Expression. Aortic banding resulted in cardiac hypertrophy, as seen in a significant increase in LV mass at both 8 and 24 weeks compared with sham animals, with no effect on body mass or

Fig. 1. Top, left ventricular mass expressed relative to tibia length. Bottom, the ratio of the mRNA for $\text{MHC}\beta$ to $\text{MHC}\alpha$.

tibia length. Dietary treatments did not significantly affect LV mass, body mass, or tibia length (Fig. 1; Table 2). The mRNA expression of ANF and the ratio of the mRNA for $\text{MHC}\beta$ to $\text{MHC}\alpha$, markers of pathological cardiac hypertrophy, were elevated in aortic-banded groups compared with sham animals at both 8 and 24 weeks (Fig. 1; Table 2). Aortic banding increased absolute wall thickness compared with sham (Table 2). Both DHA and EPA supplementation decreased LV end systolic area compared with CTRL as a main effect for diet (Table 2). Supplementation with either DHA, DHA + EPA, or EPA increased LV area fractional shortening compared with CTRL as a main effect for diet (Table 2).

Fatty Acid Composition of Mitochondrial Phospholipids. In animals fed the CTRL diet cardiac hypertrophy resulted in a decrease in DHA and total ω -3 PUFA in both SSM and IFM. In the IFM hypertrophy increased ARA and stearate in IFM only in the CTRL diet group (Figs. 2 and 3; Table 3). There were no differences between sham and hypertrophied hearts within the DHA, EPA, and DHA + EPA groups. DHA supplementation raised DHA, EPA, and the sum of DHA and EPA (Fig. 2) and decreased stearate and ARA compared with the CTRL and EPA groups. Supplementation with DHA + EPA also raised DHA, EPA, and the sum of DHA and EPA and decreased stearate and ARA compared with the CTRL and EPA groups; however, the effect was generally less than for DHA alone (Figs. 2 and 3; Table 3).

Mitochondrial Respiration and Enzyme Activities. There were no differences in respiration between sham and banded animals at 8 weeks with any of the substrates used (Table 4). At 25 weeks, there was no effect of hypertrophy on either state 3 respiration or state 4 respiration in SSM with any of the substrates tested. All three ω -3 PUFA diets reduced SSM state 3 respiration with palmitoylcarnitine + malate as a substrate compared with control diet, irrespective of surgery, with no effect on the RCR. Furthermore, hypertrophy decreased the

TABLE 2

Body and cardiac mass, tibia length, LV dimensions, and mRNA expression

Data are the mean $\pm$ SEM. $n = 8-15$.

	8 Weeks				24 Weeks								P Value for Main Effect
	CTRL				DHA				EPA				
	Sham	AAB	Sham	AAB	Sham	AAB	Sham	AAB	Sham	AAB	Sham	AAB	
Body mass (g)	490 ± 6	505 ± 11	615 ± 17	621 ± 15	652 ± 26	623 ± 18	659 ± 24	648 ± 14	675 ± 17	610 ± 21			
Tibia length (mm)	40.1 ± 0.3	40.3 ± 0.3	41.9 ± 0.6	42.43 ± 0.2	42.1 ± 0.6	42.1 ± 0.5	42.6 ± 0.3	42.6 ± 0.4	42.7 ± 0.2	41.9 ± 0.3			
LV mass (g)	0.881 ± 0.026	1.125 ± 0.080*	0.954 ± 0.022	1.223 ± 0.090	1.045 ± 0.030	1.234 ± 0.073	1.070 ± 0.047	1.179 ± 0.068	1.111 ± 0.056	1.276 ± 0.087	$P_B = 0.002$		
LV end diastolic area (cm ²)	0.753 ± 0.039	0.762 ± 0.038	0.780 ± 0.025	0.832 ± 0.041	0.755 ± 0.031	0.823 ± 0.024	0.774 ± 0.038	0.844 ± 0.037	0.860 ± 0.042	0.799 ± 0.037			
LV end systolic area (cm ²)	0.342 ± 0.029	0.352 ± 0.033	0.412 ± 0.031	0.431 ± 0.045	0.319 ± 0.029	0.335 ± 0.025#	0.323 ± 0.025	0.358 ± 0.023	0.371 ± 0.025	0.365 ± 0.033	$P_{CD} = 0.004$		
LV area of fractional shortening	0.549 ± 0.020	0.547 ± 0.028	0.474 ± 0.032	0.487 ± 0.035	0.579 ± 0.032#	0.596 ± 0.024#	0.584 ± 0.020#	0.573 ± 0.024#	0.567 ± 0.027#	0.546 ± 0.030	$P_{CD} < 0.001$		
Absolute wall thickness (cm)	0.205 ± 0.011	0.218 ± 0.011	0.204 ± 0.009	0.235 ± 0.013*	0.224 ± 0.011	0.245 ± 0.007	0.221 ± 0.010	0.230 ± 0.008	0.215 ± 0.009	0.234 ± 0.011	$P_{CD} = 0.001$		
Relative wall thickness (cm/cm)	0.524 ± 0.039	0.546 ± 0.035	0.486 ± 0.029	0.563 ± 0.041	0.590 ± 0.054	0.625 ± 0.022	0.542 ± 0.038	0.575 ± 0.029	0.518 ± 0.051	0.565 ± 0.031	$P_B = 0.007$		
MHC9/α	1.01 ± 0.11	2.13 ± 0.31*	1.01 ± 0.21	2.71 ± 1.08	0.79 ± 0.12	1.63 ± 0.47	1.22 ± 0.36	1.61 ± 0.51	1.23 ± 0.36	2.49 ± 0.74	$P_B = 0.032$		
P _{CD} : comparison between CTRL diet and DHA diet; P _{CE} : comparison between CTRL diet and EPA diet; P _{CD} : comparison between CTRL diet and DHA diet; P _{DCE} : comparison between CTRL and DHA + EPA diet; P _B :													

P_{CD} , comparison between CTRL diet and DHA diet; P_{CE} , comparison between CTRL diet and EPA diet; P_{DCE} , comparison between CTRL diet and DHA + EPA diet; P_B , comparison between sham and AAB.

*, $P \leq 0.05$ vs. respective sham; #, $P < 0.05$ vs. CTRL of respective surgery.

Fig. 2. DHA and EPA content in mitochondrial phospholipids expressed as percentage of total. BQL, below quantifiable limit. *, $p < 0.05$ compared with sham of same diet; #, $p < 0.05$ for main effect compared with CTRL group; \$, $p < 0.05$ for main effect compared with EPA group; †, $p < 0.05$ for main effect compared with DHA + EPA group.

RCR in SSM with pyruvate + malate as a substrate in the CTRL and EPA diet groups; however, RCR was maintained in the DHA and DHA + EPA groups after AAB. In IFM, hypertrophy resulted in decreased state 3 and RCR with pyruvate + malate as a substrate, and this effect was especially marked in the CTRL and EPA groups. State 3 respiration was lower in sham animals from the DHA and DHA + EPA groups compared with CTRL.

We have previously shown that mitochondrial enzyme activities are decreased in cardiac hypertrophy (Okere et al., 2006; Chess et al., 2009), and in the present study hypertrophy resulted in decreased activity of isocitrate dehydrogenase, citrate synthase, and medium-chain acyl-CoA dehydrogenase, with no significant effect of supplementation with ω -3 PUFA (Table 5).

Mitochondrial Response to Ca^{2+} . MPTP opening was not different between sham and AAB groups within any of the dietary treatments (Fig. 4). On the other hand, treatment with DHA resulted in a ~50% increase in the cumulative Ca^{2+} load needed to elicit MPTP opening in both SSM and IFM in both sham and AAB animals compared with both CTRL and EPA animals. DHA + EPA increased the cumulative Ca^{2+} load required for MPTP opening compared with CTRL in both sham and hypertrophied groups in both SSM and IFM, and EPA treatment had a similar effect but

Fig. 3. Arachidonic acid content in mitochondrial phospholipids, expressed as percentage of total. *, $p < 0.05$ compared with sham of same diet; #, $p < 0.05$ for main effect compared with CTRL group; \$, $p < 0.05$ for main effect compared with EPA group; †, $p < 0.05$ for main effect compared with DHA + EPA group.

TABLE 3

Fatty acid composition of mitochondrial phospholipids

Values are expressed as percentage of total fatty acids. Data are the mean $\pm$ SEM. $n = 5-6$.

Fatty Acid Species	CTRL		DHA		EPA		DHA + EPA		P Value for Main Effect
	Sham	AAB	Sham	AAB	Sham	AAB	Sham	AAB	
SSM									
C16:0	14.7 ± 0.5	14.9 ± 0.8	13.9 ± 0.7	14.8 ± 0.4	14.8 ± 0.4	15.9 ± 1.2	15.1 ± 0.6	15.4 ± 0.8	$P_{CD} < 0.001$ $P_{CDE} = 0.002$ $P_{DE} < 0.001$ $P_{EDE} = 0.001$ $P_{DE} = 0.004$
C18:0	22.6 ± 0.7	23.3 ± 1.5	20.0 ± 0.3\$	20.2 ± 0.4#\$	23.2 ± 0.5	22.9 ± 1.1	20.6 ± 0.5	20.4 ± 0.5#	
C18:1n9	9.9 ± 0.5	10.0 ± 0.4	9.3 ± 0.4	8.8 ± 0.3	10.5 ± 0.3	10.2 ± 0.7	9.9 ± 0.4	9.6 ± 0.5	
C18:1n7	4.8 ± 0.6	4.7 ± 0.5	3.7 ± 0.3	3.8 ± 0.4	4.5 ± 0.4	4.7 ± 0.5	4.1 ± 0.3	4.0 ± 0.4	
C18:2n6	16.5 ± 1.1	16.6 ± 1.2	18.2 ± 0.5	17.4 ± 0.7	18.0 ± 0.7	16.4 ± 1.0	17.0 ± 0.7	17.1 ± 0.7	
C20:3n6	3.1 ± 0.1	2.6 ± 0.6	2.5 ± 0.5	2.4 ± 0.5	2.2 ± 0.8	2.7 ± 0.6	2.6 ± 0.6	2.7 ± 0.6	
IFM									
C16:0	14.7 ± 0.2	15.8 ± 0.6	13.6 ± 0.6	13.9 ± 0.9	14.5 ± 0.3	15.6 ± 0.6	16.3 ± 1.4	14.9 ± 0.9	$P_{CD} < 0.001$ $P_{CDE} < 0.001$ $P_{DE} < 0.001$ $P_{EDE} < 0.001$ $P_{CD} = 0.008$ $P_{DE} = 0.009$
C18:0	22.7 ± 0.5	24.7 ± 0.8*	20.1 ± 0.6#\$	20.2 ± 0.6#\$	23.2 ± 0.6	23.5 ± 0.4†	21.6 ± 0.6	21.0 ± 0.6#	
C18:1n9	9.9 ± 0.5	10.2 ± 0.7	8.6 ± 0.7	9.0 ± 0.5	10.3 ± 0.3	9.8 ± 0.4	9.5 ± 0.5	9.1 ± 0.3	
C18:1n7	4.8 ± 0.5	4.2 ± 1.0	3.7 ± 0.4	3.7 ± 0.4	4.4 ± 0.3	5.0 ± 0.4	4.1 ± 0.3	3.9 ± 0.3	
C18:2n6	17.1 ± 1.3	18.0 ± 1.0	18.4 ± 0.6	18.1 ± 0.9	18.2 ± 0.7	17.6 ± 1.0	16.9 ± 1.3	17.8 ± 0.9	
C20:3n6	2.5 ± 0.6	1.5 ± 0.8	2.4 ± 0.6	2.4 ± 0.5	2.7 ± 0.6	1.6 ± 0.8	2.0 ± 0.7	2.5 ± 0.5	

P_{CD} , comparison between CTRL diet and DHA diet; P_{CDE} , comparison between CTRL diet and DHA + EPA diet; P_{DE} , comparison between DHA diet and EPA diet; P_{EDE} , comparison between EPA diet and DHA + EPA diet.

*, $P \leq 0.05$ vs. respective sham; #, $P < 0.05$ vs. CTRL of respective surgery; \$, $P < 0.05$ vs. EPA of respective surgery; †, $P < 0.05$ vs. DHA + EPA of respective surgery.

in SSM only (Fig. 4). There were no differences in MPTP opening between the CTRL and EPA diets. When extramitochondrial Ca^{2+} concentration was plotted as a function of the cumulative Ca^{2+} load (Fig. 5), the DHA-treated group showed a clear rightward shift compared with CTRL in sham and AAB groups in both SSM and IFM and compared with EPA in sham SSM and both sham and banded IFM. DHA + EPA also resulted in a rightward shift compared with CTRL animals in banded SSM and IFM.

Discussion

The primary novel findings of the study are: 1) DHA supplementation raised DHA in phospholipids ~ 2 -fold, increased EPA, and delayed Ca^{2+} -induced MPTP; 2) supplementation with DHA + EPA also increased DHA and delayed MPTP, but had a less profound effect on phospholipid DHA and total DHA + EPA content than DHA alone; and 3) EPA supplementation increased EPA, but did not affect DHA or MPTP opening. The positive effects of DHA and DHA + EPA were evident in both normal and hypertrophied myocardium. Taken together, these results show that treatment with DHA alone effectively increases both DHA and EPA in cardiac mitochondrial phospholipids and delays MPTP, and treat-

ment with DHA + EPA offers no further advantage over DHA alone.

Although all three ω -3 PUFA diets increased mitochondrial phospholipid total DHA + EPA and depleted ARA, the effect was significantly greater with DHA than DHA + EPA or EPA. We have postulated that the delay in MPTP opening with supplementation with either DHA + EPA or DHA may be caused by depletion of ARA (O'Shea et al., 2009; Khairallah et al., 2010). Previous studies implicated ARA in the triggering of MPTP opening, because inhibition of ARA release from membranes protects mitochondria from MPTP in liver, heart, and kidneys (Williams and Gottlieb, 2002; Penzo et al., 2004; Kinsey et al., 2007). The present results suggest that changes in ARA do not necessarily predict a delay in Ca^{2+} -induced MPTP opening, because treatment with EPA caused a significant decrease in ARA content but had no effect on MPTP opening. This suggests that the delay in Ca^{2+} -induced MPTP opening seen with DHA and DHA + EPA supplementation is solely attributable to elevated membrane DHA, not ARA depletion. Although supplementation with EPA seems ineffective and the beneficial effect of DHA + EPA seems to be caused by incorporation of DHA into membrane phospho-

TABLE 4
Mitochondrial parameters and the activity of mitochondrial enzyme in cardiac tissue
Data are the mean ± SEM. *n* = 8–15.

	8 weeks						24 Weeks						P Value for Main Effect
				CTRL			DHA			EPA			
	Sham	AAB		Sham	AAB		Sham	AAB		Sham	AAB		
SSM													
State 3 (nA O · mg ⁻¹ · min ⁻¹)													
Pyruvate	259 ± 24	250 ± 17		237 ± 24	177 ± 20		159 ± 19	201 ± 17		180 ± 28	171 ± 24		159 ± 19
Palmitoylecarnitine	187 ± 26	198 ± 21		227 ± 15	196 ± 12		154 ± 24#	158 ± 14		120 ± 26#	131 ± 12#		137 ± 11#
													$P_{CD} = 0.002$
													$P_{CE} < 0.001$
													$P_{CDE} < 0.001$
Succinate	323 ± 21	312 ± 16		305 ± 25	264 ± 15		263 ± 29	237 ± 27		244 ± 22	289 ± 22		266 ± 22
State 4 (nA O · mg ⁻¹ · min ⁻¹)													
Pyruvate	51 ± 13	51 ± 10		32 ± 4	50 ± 13		32 ± 3	41 ± 12		26 ± 4	40 ± 4		34 ± 5
Palmitoylecarnitine	37 ± 5	42 ± 8		37 ± 6	34 ± 5		29 ± 5	29 ± 4		20 ± 6	29 ± 4		26 ± 2
Succinate	70 ± 6	85 ± 10		108 ± 16	99 ± 11		94 ± 11	89 ± 10		72 ± 10	93 ± 11		103 ± 6
RCR													
Pyruvate	7.4 ± 2.0	5.6 ± 0.7		8.0 ± 1.2	5.0 ± 0.3*		5.4 ± 0.8	7.0 ± 0.7		8.2 ± 1.5	4.6 ± 0.7**\$		5 ± 0.6
Palmitoylecarnitine	6.2 ± 1.7	6.0 ± 1.2		7.0 ± 1.1	8.2 ± 1.6		6.2 ± 1.5	6.1 ± 0.6		7.8 ± 1.7	5.2 ± 0.6		5.7 ± 0.5
Succinate	4.7 ± 0.3	4.0 ± 0.3		3.0 ± 0.2	2.9 ± 1.0		2.9 ± 0.4	2.7 ± 0.2		3.9 ± 0.6	3.8 ± 0.5\$		3 ± 0.1£
													$P_{CE} = 0.027$
													$P_{DE} = 0.007$
													$P_{EDE} = 0.001$
IFM													
State 3 (nA O · mg ⁻¹ · min ⁻¹)													
Pyruvate	307 ± 32	268 ± 27		296 ± 13	217 ± 19*		223 ± 30#	192 ± 16		266 ± 30	195 ± 23*		216 ± 20
Palmitoylecarnitine	259 ± 24	269 ± 20		233 ± 29	171 ± 16		143 ± 17	160 ± 19		161 ± 19	171 ± 16		156 ± 21
Succinate	403 ± 27	380 ± 26		356 ± 31	366 ± 22		310 ± 38	320 ± 28		312 ± 23	344 ± 18		321 ± 18
State 4 (nA O · mg ⁻¹ · min ⁻¹)													
Pyruvate	89 ± 26	64 ± 9		49 ± 4	45 ± 4		40 ± 5	42 ± 3		44 ± 7	50 ± 4		51 ± 4
Palmitoylecarnitine	49 ± 8	59 ± 12		36 ± 7	34 ± 7		32 ± 5	32 ± 4		28 ± 8	38 ± 7		37 ± 5
Succinate	106 ± 12	91 ± 8		141 ± 16	132 ± 11		107 ± 11	119 ± 10		99 ± 12	113 ± 9		125 ± 5
RCR													
Pyruvate	4.9 ± 1.1	5.0 ± 1.1		6.4 ± 0.6	5.1 ± 0.6		5.6 ± 0.5	4.8 ± 0.5		6.8 ± 0.9	4.0 ± 0.5*		4.5 ± 0.5
Palmitoylecarnitine	6.5 ± 1.1	5.9 ± 0.9		7.7 ± 1.4	6.6 ± 1.0		5.0 ± 0.6	7.2 ± 2.1		7.4 ± 1.3	5.8 ± 1.0		4.4 ± 0.4
Succinate	4.0 ± 0.4	4.3 ± 0.1		2.7 ± 0.2	2.8 ± 0.1		3.2 ± 0.2	2.8 ± 0.2		3.5 ± 0.4#	3.2 ± 0.2		2.8 ± 0.1
													$P_B < 0.001$
													$P_{CE} = 0.006$
													$P_{EDE} = 0.031$
Mitochondrial yield (mg/gww)													
SSM	20.59 ± 0.50	20.53 ± 0.85		19.76 ± 1.30	18.61 ± 1.17		21.34 ± 1.30	22.28 ± 1.01		23.95 ± 1.77	22.77 ± 1.77		22.74 ± 1.41
IFM	9.55 ± 0.88	9.26 ± 0.53		13.36 ± 0.69	11.81 ± 0.81		12.93 ± 1.11	12.87 ± 0.69		13.00 ± 0.66	12.22 ± 0.58		12.06 ± 0.77
													$P_B = 0.041$

*P*_{CD}, comparison between CTRL diet and DHA diet; *P*_{CE}, comparison between CTRL diet and EPA diet; *P*_{CDE}, comparison between CTRL and DHA + EPA diets; *P*_{DE}, comparison between EPA and DHA + EPA diets; *P*_B, comparison between sham and AAB.
*, *P* < 0.05 vs. respective sham of same diet; #, *P* < 0.05 vs. CTRL of same surgery.

TABLE 5

Mitochondrial enzyme activities from whole tissue homogenates

Data are the mean $\pm$ SEM. $n = 8$ –15.

	CTRL		DHA		EPA		DHA + EPA		<i>P</i> Value for Main Effect
	Sham	AAB	Sham	AAB	Sham	AAB	Sham	AAB	
Isocitrate dehydrogenase (mmol $\cdot$ min ⁻¹ $\cdot$ g ⁻¹)	46.8 $\pm$ 2.3	43.5 $\pm$ 2.7	42.8 $\pm$ 1.5	43.6 $\pm$ 1.4	52.3 $\pm$ 3.3	46.2 $\pm$ 2.1	47.7 $\pm$ 2.5	42.2 $\pm$ 1.7	$P_B = 0.03$
Medium-chain acyl-CoA dehydrogenase (μ mol $\cdot$ min ⁻¹ $\cdot$ g ⁻¹)	13.1 $\pm$ 0.4	11.9 $\pm$ 0.7	13.8 $\pm$ 0.5	12.0 $\pm$ 0.6	14.0 $\pm$ 0.4	12.6 $\pm$ 0.5	13.9 $\pm$ 0.6	12.7 $\pm$ 1.0	$P_{DE} = 0.039$ $P_B = 0.007$
Citrate synthase (μ mol $\cdot$ min ⁻¹ $\cdot$ g ⁻¹)	126.6 $\pm$ 6.4	121.6 $\pm$ 5.5	119.3 $\pm$ 3.0	116.8 $\pm$ 3.9	140.1 $\pm$ 7.9	119.0 $\pm$ 6.2	125.8 $\pm$ 4.9	110.4 $\pm$ 4.4	$P_B = 0.006$

 P_{DE} , comparison between DHA and EPA diets; P_B , comparison between sham and AAB.*, $P < 0.05$ vs. respective sham of same diet.

Fig. 4. The cumulative Ca^{2+} load required for MPTP opening. *, $p < 0.01$ for main effect compared with CTRL and EPA groups; †, $p < 0.02$ for main effect compared with CTRL group. a, $p < 0.05$ compared with sham CTRL; b, $p < 0.02$ compared with sham EPA; c, $p < 0.01$ compared with AAB CTRL; d, $p < 0.01$ compared with AAB EPA; e, $p < 0.03$ compared with AAB CTRL.

lipids, it is important to note that the DHA + EPA group received 50% of the DHA given to the DHA-only group. To fully exclude an effect of EPA, future studies should compare DHA alone with DHA + EPA over a wide range of equivalent doses of DHA.

The GISSI-HF trial (GISSI-Hf Investigators, 2008) showed a significant decrease in cardiovascular events and death in patients with heart failure with a low dose of ω -3 PUFA by using a formulation containing DHA and EPA at a 55:45 ratio. Likewise, we showed that treatment with DHA and EPA at a 70:30 ratio prevented LV remodeling and cardio-

myocyte apoptosis in rats subjected to aortic banding (Duda et al., 2009b). Neither study compared treatment with combined DHA + EPA to monotherapy with DHA or EPA. The present study is the first to compare the effects of EPA, DHA, and DHA + EPA on mitochondrial phospholipids, function, and MPTP opening. Our results show that inclusion of EPA in ω -3 PUFA formulations that are high in DHA is not essential for the beneficial effect of DHA in preventing Ca^{2+} -induced MPTP. Furthermore, our findings provide some evidence that DHA may be superior to an equivalent amount of DHA + EPA, because DHA resulted in a greater increase in total DHA + EPA and DHA alone and decrease in ARA. The cumulative Ca^{2+} load necessary to induce MPTP opening was positively correlated with DHA content in mitochondrial phospholipid ($r = 0.38$, $p < 0.001$, $n = 47$), but not EPA content ($r = 0.08$, $p = 0.46$), which suggests that elevated DHA is the critical factor and EPA does not contribute to the prevention of MPTP opening. Additional studies are needed with a wide range of DHA and EPA doses to determine how much of an increase in DHA is necessary to affect MPTP opening and whether extremely high doses of EPA can have a similar effect.

Epidemiological studies show that cardiac hypertrophy is a strong predictor for the development of heart failure. In the present study aortic-banded animals showed signs of early cardiac pathology, LV chamber enlargement, and metabolic dysfunction (e.g., increase in mRNA for ANF and MHC β /MHC α , LV end systolic area, and suppressed activity of mitochondrial oxidative enzymes). Future studies should assess the effects of DHA and EPA in models of more advanced heart failure, with dilated LV end diastolic volume, reduced ejection fraction, and accelerated cell death and fibrosis, which do not occur in the relatively mild form of pathological LV hypertrophy used in the present investigation.

In summary, our results show clear differences between supplementation with DHA and the more commonly used formulations containing a mixture of DHA and EPA. Monotherapy with DHA doubled the DHA content of mitochondrial phospholipids, increased EPA, and delayed Ca^{2+} -induced MPTP, whereas treatment with DHA + EPA also increased DHA and delayed MPTP, but did not have such a profound effect on DHA and total DHA + EPA content in mitochondrial phospholipids. These findings suggest that treatment with DHA + EPA offers no further advantage over DHA alone. Based on the favorable results with the GISSI-HF trial (GISSI-Hf Investigators, 2008), future clinical studies should

Fig. 5. Extramitochondrial Ca^{2+} concentration plotted as a function of the cumulative Ca^{2+} load.

determine whether DHA is superior to DHA + EPA in affecting clinical outcomes.

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