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Differential effects of n-3 polyunsaturated fatty acids on metabolic control and vascular reactivity in the type 2 diabetic *ob/ob* mouse

Vikkie A. Mustad[a,*], Stephen DeMichele[a], Yung-Sheng Huang[a], Amanda Mika[b], Nathan Lubbers[b], Nathalie Berthiaume[b], Jim Polakowski[b], Brad Zinker[b]

[a]*Healthy Living Research and Development, Ross Products Division, Abbott Laboratories, Columbus, OH 43215, USA*
[b]*Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL 60064, USA*

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Abstract

Diets rich in monounsaturated fatty acids (MUFA) are recommended for individuals with type 2 diabetes mellitus (T2DM). The American Heart Association recommends increasing intakes of n-3 polyunsaturated fatty acids (PUFA) to reduce the risk of vascular disease in high-risk individuals; however, the long-term effects of these bioactive fatty acids on glucose metabolism in insulin resistance are controversial. The present studies were conducted to evaluate the effects of diets rich in both MUFA and α linolenic acid (C18:3n-3, ALA), eicosapentaenoic acid (C20:5n-3, EPA), or docosahexaenoic acid (C22:6n-3, DHA), on glycemic control and other parameters related to vascular health in a mouse model of T2DM and insulin resistance. Male *ob/ob* mice (n = 15 per treatment) were fed 1 of 4 lipid-modified formula diets (LFDs) for 4 weeks: (1) MUFA control, (2) ALA blend, (3) EPA blend, and (4) DHA blend. A portion of a MUFA-rich lipid blend in the control LFD was replaced with 11% to 14% energy as n-3 PUFA. After 4 weeks, plasma glucose response to a standard meal (1.5 g carbohydrate/kg body weight) and insulin challenge (2 U/kg body weight, IP) was assessed, and samples were collected for analysis of glucose, insulin, and lipids. Vascular reactivity of isolated aortic rings was assessed in an identical follow-up study. The results showed that insulin-resistant mice fed an LFD with EPA and/or DHA blends had significantly ($P < .05$) lower triglycerides and free fatty acids, but insulin sensitivity and fasting plasma glucose were not improved. However, mice fed with the ALA blend had significantly improved insulin sensitivity when compared to those fed with other LFD ($P < .05$). Animals fed an LFD with n-3 PUFA from marine or plant sources showed significantly improved vascular responses as compared with the MUFA-rich LFD (E_{max}, $P < .05$) and *ob/ob* reference mice consuming chow (E_{max} and pEC_{50}, $P < .05$). In summary, long-term consumption of LFD with n-3 PUFAs improved blood lipids and vascular function in an animal model of insulin resistance and T2DM; however, only MUFA-rich LFD with ALA also improved both insulin sensitivity and glycemic responses. Further studies of MUFA-rich LFD with ALA with individuals who have T2DM are warranted.

1. Introduction

The optimal dietary fat and fatty acid composition for individuals with type 2 diabetes mellitus (T2DM) is controversial. Low-fat diets rich in carbohydrate may worsen plasma glucose, insulin, triglycerides, and plasma high-density lipoprotein cholesterol levels [1]. In comparison to these diets, those rich in monounsaturated fatty acids (MUFA) improve blood lipids and glucose homeostasis [1-5]. As a result, the American Diabetes Association has recommended MUFA-rich diets for T2DM [6]. However, diabetes is now considered a cardiovascular disease equivalent [7], and intakes of n-3 PUFA have been recognized as playing an important role in the prevention and treatment of vascular disorders in high-risk individuals [8]. Indeed, analyses of trials with individuals with T2DM [9,10] reveal that supplementation with marine oil n-3 PUFA improves hypertriglyceridemia and other important cardiovascular risk factors in this population.

Studies in rodents and other animal models have shown that insulin resistance can be improved, delayed, or prevented when fat-rich diets contain marine oil n-3 PUFA [10-14]. However, the results of human intervention studies have reported both improvements in and worsening of glycemic control and insulin sensitivity when individuals with insulin

* Corresponding author. Tel.: +1 614 624 6355; fax: +1 614 624 3705.
E-mail address: vikkie.mustad@abbott.com (V.A. Mustad).

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resistance or T2DM are supplemented with these fatty acids [9,10]. As a result, recommendations for dietary marine oil n-3 PUFA for individuals with T2DM are controversial and have been tempered because of concerns over the long-term effects of these fatty acids on glycemic control [11]. Recently, the cardiovascular benefits of the plant source of the 18-carbon n-3 PUFA, α-linolenic acid (C18:3n-3), have also been recognized [8]; however, the differential effects of both marine and plant n-3 PUFA on glycemic control and vascular health inT2DM have not been evaluated in a single study.

The purpose of this study, therefore, was to evaluate the effects of long-term feeding of MUFA-rich diets containing n-3 PUFA of different source and chain lengths (ie, C18, C20, C22) and number of double bonds (ie, 3, 5, or 6) on glucose metabolism, insulin sensitivity, blood lipids, and vascular reactivity in the *ob/ob* mouse, a model with key features of the metabolic syndrome including T2DM and insulin resistance.

2. Methods

2.1. Animals and experimental design

Male mice (*ob/ob*), 4 to 5 weeks of age (~ 40 g body weight), purchased from Jackson Laboratory (Bar Harbor, ME), were maintained throughout the study in the animal care facility (barrier isolated laboratory) of the Global Pharmaceutical Research Development Division of Abbott Laboratories (Abbott Park, IL). Animals were treated in conformity with the Abbott Laboratories Institutional Animal Care and Use Committee guidelines. Mice were housed 5 per cage with free access to food and water. Animals were immediately placed on the control liquid formula (see description below) and allowed to acclimatize to the feeding and facilities for 1 week. Animals were then randomized (based on postprandial plasma glucose and body weight) to 1 of the 4 experimental lipid-modified formula diet (LFD) groups (n = 15 mice per group; see description below and Table 1); an additional chow reference diet group (n = 15 mice) was included for comparison of food intake and growth only. The LFD was provided to the animals through graduated glass drinking bottles, and fresh diet was provided every 24 hours. Individual animals were weighed twice per week throughout the experimental period. Blood samples were collected weekly at the same time each day by tail snip to measure fed plasma glucose and insulin levels. After 26 days on the experimental LFD, standardized meal tolerance test (MTT; n = 10) and insulin tolerance test (ITT; n = 5) were administered. Animals were then returned to their respective experimental diets and killed on day 29. Blood samples were obtained from all animals at this time for analysis of postprandial glucose, insulin, triglycerides, free fatty acids, and total glycated hemoglobin. Samples of adipose, liver, skeletal muscle, and aortic tissues were excised and immediately frozen in liquid nitrogen for fatty acid analysis.

Table 1
Fatty acid composition of experimental formula diets

Fatty acids (g/100 g)	Control MUFA blend	ALA blend	EPA blend	DHA blend
C14:0	0.1	0.1	2.8	2.4
C16:0	5.4	5.9	7.0	13.5
C18:0	0.4	0.7	1.4	3.4
Total SFA	6.8	7.3	14.1	20.8
C16:1n-7	0.1	0.1	4.5	3.3
C18:1n-7	–	–	1.3	1.7
C18:1n-9	74.1	49.5	16.1	13.3
C20:1n-9	0.4	0.3	0.4	1.1
C22:1n-9	–	–	0.1	0.2
Total MUFA	75.0	50.1	22.9	22.4
C18:2n-6	17.0	18.0	23.8	18.6
C18:3n-6	–	–	–	0.1
C20:3n-6	–	–	0.1	0.1
C20:4n-6	–	–	0.8	1.3
C22:5n-6	–	–	0.2	0.7
Total n-6	17.0	18.0	25.0	21.4
C18:3n-3	1.2	24.6	0.8	0.8
C18:4n-3	–	–	2.2	1.2
C20:3n-3	–	–	0.1	0.2
C20:4n-3	–	–	0.6	0.5
C20:5n-3	–	–	20.9	9.0
C22:5n-3	–	–	2.0	1.4
C22:6n-3	–	–	9.3	21.4
Total n-3	1.2	24.6	35.8	34.4
MUFA/n-3 ratio	60.0	2.0	0.5	0.4
n-6/n-3 ratio	13.6	0.7	0.7	0.6

Table 2
Weekly body weight, plasma glucose, and insulin values in *ob/ob* mice fed with LFD

	MUFA control	ALA blend	EPA blend	DHA blend
Body weight (g)				
Week 0	37.4 ± 1.0	37.4 ± 1.2	38.2 ± 0.9	40.2 ± 1.1
Week 1	43.0 ± 0.9	43.8 ± 1.0	41.3 ± 0.7	43.3 ± 1.1
Week 2	49.2 ± 0.7	50.6 ± 0.9	47.7 ± 0.8	49.2 ± 0.9
Week 3	52.9 ± 0.8	54.2 ± 0.8	51.9 ± 0.7	53.1 ± 0.9
Week 4	56.1 ± 0.9	56.0 ± 1.0	54.2 ± 0.7	55.9 ± 0.8
Glucose (mg/dL)				
Week 0	251 ± 19	249 ± 18	247 ± 12	251 ± 15
Week 1	249 ± 14	257 ± 9	262 ± 9	252 ± 13
Week 2	255 ± 17	254 ± 12	292 ± 15	289 ± 11
Week 3	229 ± 16	221 ± 11	265 ± 14	273 ± 15
Week 4	293 ± 25	314 ± 13	340 ± 12	343 ± 14
Insulin (ng/dL)				
Week 0	17.2 ± 11	10.4 ± 3	12.5 ± 4	11.5 ± 2
Week 1	20.9 ± 4[a]	22.9 ± 3[a]	50.2 ± 11[b]	26.7 ± 7[a]
Week 2	36.6 ± 9[a]	30.8 ± 6[a]	81.3 ± 11[b]	35.3 ± 9[a]
Week 3	14.0 ± 2[a]	28.3 ± 6[b]	64.3 ± 11[c]	38.4 ± 10[b]
Week 4	11.1 ± 3[a]	17.6 ± 4[a]	37.0 ± 8[b]	17.3 ± 4[a]

Values are expressed as mean ± SEM (n = 15 animals per treatment). Animals were weighed twice per week and values reflect the weekly average. Blood for glucose and insulin was taken from nonfasted animals at approximately the same time of day. [abcd]$P < .05$, means in a row with different superscript letters are significantly different.

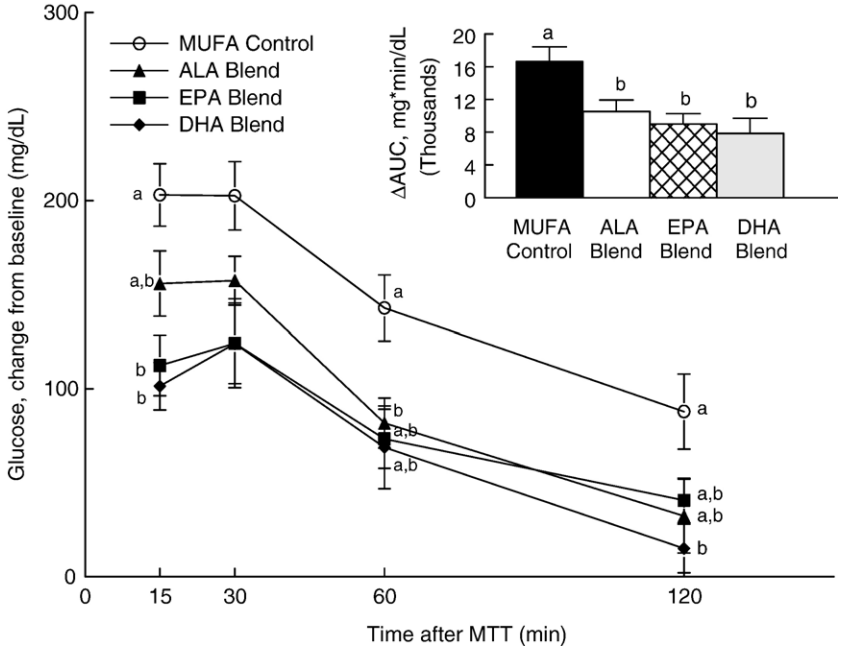

Fig. 1. Glucose response, change from baseline, and ΔAUC (mean ± SEM) after standard MTT in *ob/ob* mice consuming LFD (n = 10 animals per treatment). $^{ab}P < .05$, means at each time point having different superscript letters are significantly different.

2.2. Diets

A nutritionally complete diabetic nutritional (Glucerna Ross Products Division, Abbott Laboratories, Columbus, OH) was used as the base formula for this study. The formulas were similar in content of protein (18% kJ; casein and soy protein), carbohydrate (37%-39% kJ; maltodextrin and fructose), fiber (0.002 g/J; soy fiber), and total fat (43%-45% kJ), but differed according to lipid blend (Table 1). The MUFA control (85% high-oleic safflower oil [HOSO], 10% canola oil, 5% lecithin) was rich in C18:1n-9 with a low level of n-3 PUFA. The ALA blend (50% HOSO, 39% flaxseed oil, 6% corn oil, 5% lecithin) was rich in both MUFA and C18:3n-3. The eicosapentaenoic acid (EPA) blend (58% sardine oil, 26% safflower oil, 11% HOSO) was rich in C20:5n-3, whereas the docosahexaenoic acid (DHA) blend (65% tuna oil, 21% safflower oil, 9% sardine oil, 5% lecithin) was rich in C22:6n-3. All three n-3 PUFA-

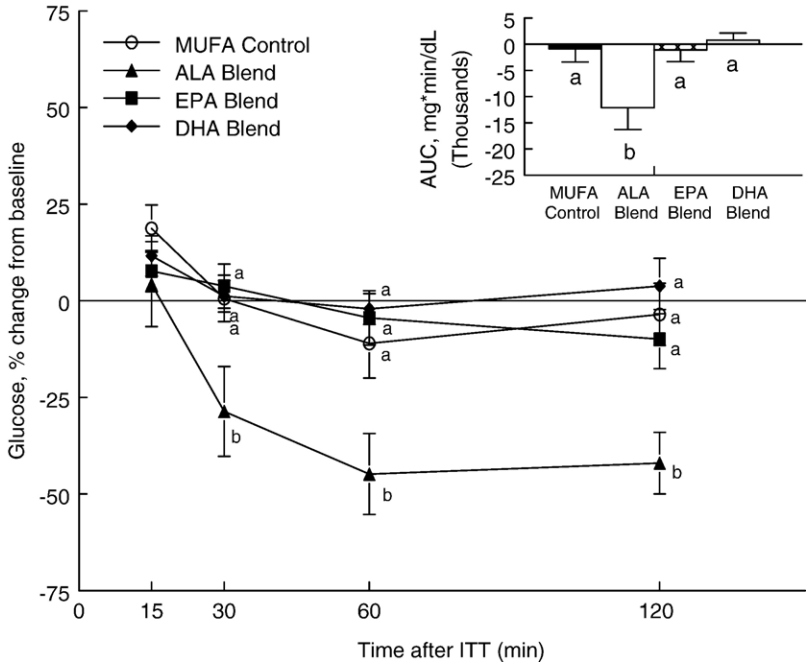

Fig. 2. Glucose response, percent change from baseline, and ΔAUC (mean ± SEM) after standard ITT in *ob/ob* mice consuming LFD (n = 5 animals per treatment). $^{ab}P < .05$, means at each time point having different superscript letters are significantly different.

containing LFD had a similar ratio of n-6/n-3 fatty acids, although the MUFA/n-3 ratio differed (Table 1).

2.3. Meal tolerance test

Meal tolerance tests were performed after 26 days of feeding to assess postprandial glucose metabolism. Mice (n = 10 per group) were fasted for 3 hours; a 0-minute baseline blood sample was taken for glucose analyses. Animals were then gavaged with the MTT formula (Ensure Plus Vanilla, Abbott Laboratories, Columbus, OH), and additional samples were taken at 15, 30, 60, and 120 minutes postgavage. The MTT dose was given to provide 1.5 g carbohydrate/kg body weight.

2.4. Insulin tolerance test

An ITT was performed after 26 days of feeding in the remaining mice (n = 5 per diet group) that did not undergo the MTT. Mice were fasted for 3 hours; a 0-minute baseline blood sample was taken for glucose analyses. The animals were then injected with insulin IP at a dose of 2 U/kg body weight (Humulin-R in 0.1% bovine serum albumin, Lilly, Indianapolis, IN), and additional blood samples were taken at 15, 30, 60, and 120 minutes postinjection.

2.5. Processing and analysis of blood and tissue samples

After 28 days, mice were anesthetized by asphyxiation with dry ice, and blood samples were drawn by cardiac puncture. Plasma was separated by centrifugation at 4°C and assayed immediately (glucose) or stored at −20°C for subsequent analysis of other hormones and lipid variables (see below). After animals were killed, aortic, liver, adipose, and hind limb skeletal muscle tissues were removed, rinsed briefly in saline, blotted, and immediately frozen at −70°C in freezer-safe bags flushed with nitrogen for subsequent analysis. Composition of phospholipid fatty acids was analyzed using gas chromatography using a HP Model 5890 series II plus gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with an Omegawax 320 fused silica capillary column (0.32 mm × 30 m × 0.25 μm; Supelco, Bellefonte, PA). Plasma glucose was measured by the glucose oxidase method by use of the Precision G glucose monitor system (Abbott Laboratories, Abbott Park,

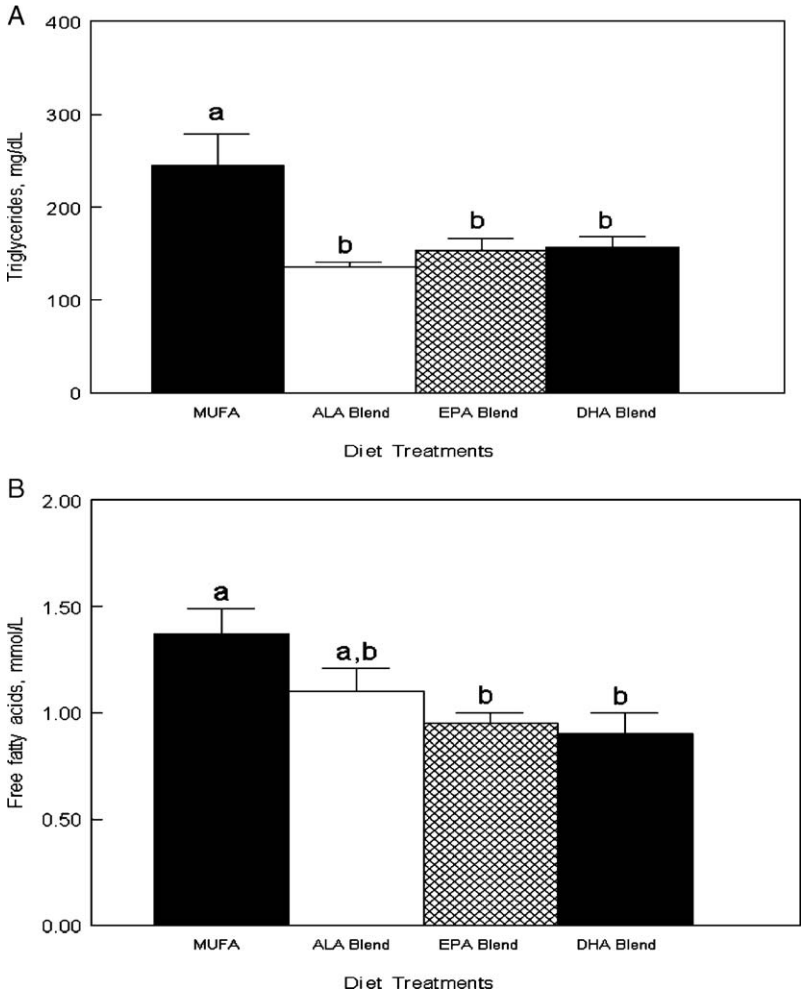

Fig. 3. Serum levels (mean ± SEM) of (A) triglycerides and (B) free fatty acids in *ob/ob* mice consuming LFD (n = 15 animals per treatment). $^{ab}P < .05$, means with different superscript letters are significantly different.

Table 3
Fatty acid composition of liver phospholipids from *ob/ob* mice fed with LFD

Fatty acids (rel %)	Control MUFA blend	ALA blend	EPA blend	DHA blend
C14:0	0.02 ± 0.02[a]	0.02 ± 0.01[a]	0.11 ± 0.005[b]	0.12 ± 0.004[c]
C16:0	15 ± 0.4[a]	16 ± 0.3[b]	21 ± 0.3[c]	19 ± 0.2[d]
C18:0	26 ± 0.7[a]	25 ± 1[a]	30 ± 0.2[b]	27 ± 0.7[a]
Total SFA	41 ± 0.7[a]	41 ± 1.2[a]	52 ± 0.3[b]	46 ± 0.2[c]
C16:1n-7	0.4 ± 0.02[a]	0.4 ± 0.02[a]	0.7 ± 0.02[b]	0.5 ± 0.02[a]
C18:1n-7	2.3 ± 0.1[a]	1.9 ± 0.04[b]	1.7 ± 0.04[c]	1.2 ± 0.02[d]
C18:1n-9	11.8 ± 0.2[a]	10.8 ± 0.8[a]	7.9 ± 0.3[b]	6.1 ± 0.2[c]
C20:1n-9	0.63 ± 0.04[a]	0.60 ± 0.08[a]	0.20 ± 0.01[b]	0.20 ± 0.01[b]
C22:1n-9	0.29 ± 0.04[a,b]	0.34 ± 0.05[a,b]	0.31 ± 0.03[b]	0.23 ± 0.01[a,c]
Total MUFA	15.9 ± 0.2[a]	14.3 ± 0.7[a]	11 ± 0.3[b]	8 ± 0.2[c]
C18:2n-6	9.5 ± 0.1[a]	15.2 ± 1.8[b]	10.5 ± 0.15[c]	8.8 ± 0.14[d]
C18:3n-6	0.18 ± 0.03[a]	0.13 ± 0.03[a]	0.08 ± 0.01[b]	0.03 ± 0.01[c]
C20:3n-6	3.4 ± 0.1[a]	2.6 ± 0.2[a]	1.3 ± 0.02[b]	1.2 ± 0.04[b]
C20:4n-6	18.5 ± 0.3[a]	9.6 ± 0.6[b]	6.0 ± 0.1[c]	10.4 ± 0.1[b]
C22:5n-6	0.03 ± 0.05[a]	0.08 ± 0.02[b]	0.11 ± 0.01[b,c]	0.26 ± 0.01[d]
Total n-6	33 ± 0.4[a]	28 ± 1.0[b]	18 ± 0.2[c]	21 ± 0.2[d]
C18:3n-3	0.1 ± 0.03[a]	1.0 ± 0.2[b]	0.03 ± 0.02[a]	0.2 ± 0.02[a]
C18:4n-3	0.01 ± 0.01[a]	0.11 ± 0.02[b]	–	–
C20:3n-3	0.14 ± 0.04[a]	0.44 ± 0.07[b]	0.05 ± 0.01[c]	0.09 ± 0.01[a,c]
C20:4n-3	0.14 ± 0.04[a]	0.49 ± 0.05[b]	0.26 ± 0.01[c]	0.24 ± 0.01[c]
C20:5n-3	0.7 ± 0.01[a]	4.2 ± 0.30[b]	7.2 ± 0.17[c]	6.4 ± 0.14[d]
C22:5n-3	0.57 ± 0.07[a]	1.2 ± 0.08[b]	1.1 ± 0.03[b]	0.87 ± 0.02[c]
C22:6n-3	7.7 ± 0.2[a,b]	7.3 ± 0.5[a]	8.9 ± 0.3[b]	16.0 ± 0.2[d]
Total n-3	9.4 ± 0.32[a]	14.6 ± 0.66[b]	17.5 ± 0.54[c]	23.8 ± 0.31[d]
MUFA/n-3 ratio	1.69	0.98	0.62	0.34
n-6/n-3 ratio	3.5	1.91	1.02	0.88

Individual fatty acid values are expressed as mean ± SEM (n = 13-15 animals per treatment). rel % indicates relative percent. [abcd]$P < .05$, means in a row with different superscript letters are significantly different.

IL). Plasma insulin, free fatty acids, and triglycerides were measured by enzyme-linked immunosorbent assay (ALPCO Diagnostics, Windham, NH). Glycated hemoglobin was measured using a boronate affinity column via high-performance liquid chromatography (Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL).

2.6. Vascular reactivity assessment

A separate cohort of male *ob/ob* mice (6-7 weeks of age), feeding the same experimental LFD (MUFA control, ALA blend, and EPA blend, and under the same experimental conditions as described previously, was used to measure vascular reactivity. The exception was that the DHA blend diet was omitted because the previous study showed no differences in responses between the DHA and EPA blends. In addition, *ob/ob* and lean littermate mice consuming standard pelleted chow were included as a reference comparison. After 28 days of feeding, mice (n = 8 animals per treatment) were anesthetized with Nembutal (Abbottt Laboratories, Abbott Park, IL), and thoracic aortas were carefully isolated, excised, and immediately placed in buffering solution. Under ×2 magnification the aortas were cleared of fat and connective tissue, cut into rings of approximately 1 to 2 mm, and suspended in 10-mL tissue baths under 0.5 g of resting tension. Extreme care was exercised to minimize damage to the endothelium. Tension was recorded with Grass FT03 isometric transducers (Astro-Med, West Warwich, RI) connected to a Grass 7D polygraph and a Ponemah data acquisition system (Data-Sciences Intl, St. Paul, MN). The tissues were bathed in a modified Krebs solution containing (in mmol/L) 120 NaCl, 20 $NaHCO_3$, 11 dextrose, 4.7 KCl, 2.5 $CaCl_2$, 1.5 $MgSO_4$, 1.2 KH_2PO_4, and equilibrated with 5% CO_2–95% O_2 (pH 7.4 at 37°C). The tissues were equilibrated for 60 to 90 minutes with rinsing of the tissue at 10-minute intervals.

Aortic rings were precontracted with phenylephrine (PE, 10^{-5} mol/L) to produce endothelium-dependent or endothelium-independent relaxation, and cumulative concentration-effect curves to carbachol (CARB) or sodium nitroprusside (SNP) were constructed. Each constriction induced by PE was used as the internal standard for calculation of CARB- or SNP-induced relaxation as a percentage of the PE-induced precontraction. From each artery segment, the actual maximal relaxation (E_{max}) was determined and the concentration of the agonist that produced 50% of the maximal response (EC50) was calculated by nonlinear regression curve fitting (GraphPad Prism, San Diego, CA) and reported as the positive logarithm of the EC50 (pEC_{50}).

2.7. Statistical methodology

The statistical analysis was done using 1-way analysis of variance, and if treatment effects were significant, Tukey honestly significant difference test was used to determine significant pairwise treatment differences. Differences after MTT were analyzed with respect to each time point (adjusted to baseline), adjusted area under the curve (ΔAUC), adjusted peak response, and time to peak response. Differences among groups after ITT were analyzed with respect to each time point (adjusted to baseline), negative ΔAUC, adjusted negative peak response (nadir), and time to nadir. A result of a test was statistically significant if the P value was less than .05. Statistical differences for the vascular reactivity curves were determined by analysis of variance with a post hoc Newman-Keuls or unpaired Student t test, as determined by experimental design (Statview 5.0.1, SAS Institute, Cary, NC).

3. Results

3.1. Weekly body weight, plasma glucose, and insulin levels

There were no significant differences in changes in body weight in animals consuming LFD with different n-3 blends as compared with animals consuming the control MUFA blend or compared with those receiving the chow diets throughout the 4-week study (Table 2). There were no significant differences in weekly plasma glucose levels among groups consuming the LFD, although there was a

Table 4
Fatty acid composition of skeletal muscle phospholipids from *ob/ob* mice fed with LFD

Fatty acids (rel %)	Control MUFA blend	ALA blend	EPA blend	DHA blend
C14:0	0.3 ± 0.06[a]	0.4 ± 0.02[a]	0.9 ± 0.04[b]	0.5 ± 0.05[a]
C16:0	17.8 ± 0.6[a]	19.7 ± 0.3[b]	25.3 ± 0.6[c]	24.0 ± 0.4[c]
C18:0	16.9 ± 0.20[a]	16.0 ± 0.16[a]	16.2 ± 0.26[a]	19.0 ± 0.34[b]
Total SFA	36 ± 0.16[a]	37 ± 0.16[a]	43 ± 0.16[b]	44 ± 0.16[b]
C16:1n-7	0.7 ± 0.02[a]	0.9 ± 0.01[a,b]	2.1± 0.12[c]	1.1 ± 0.05[b]
C18:1n-7	3.2 ± 0.08[a]	2.8 ± 0.03[b]	3.3 ± 0.06[a]	3.2 ± 0.4[a]
C18:1n-9	16.1 ± 0.5[a]	11.7 ± 0.25[b]	8.3 ± 0.5[c]	6.2 ± 0.16[d]
C20:1n-9	0.4 ± 0.01[a]	0.2 ± 0.004[b]	0.2 ± 0.01[b]	0.2 ± 0.01[b]
C22:1n-9	0.6 ± 0.02[a]	0.4 ± 0. 02[b]	0.25 ± 0.02[c]	0.15 ± 0.01[d]
Total MUFA	21.1 ± 0.4[a]	16.1 ± 0.2[b]	14.4 ± 0.7[c]	11.1 ± 0.2[d]
C18:2n-6	10.8 ± 0.4[a]	11.8 ± 0.4[a]	8.0 ± 0.5[b]	6.0 ± 0.2[c]
C20:3n-6	1.6 ± 0.03[a]	1.4 ± 0.02[b]	0.4 ± 0.01[c]	0.4 ± 0.01[c]
C20:4n-6	10.6 ± 0.2[a]	6.8 ± 0.1[b]	3.5 ± 0.2[c]	4.3 ± 0.1[c]
C22:5n-6	0.5 ± 0.02[a]	0.2 ± 0.01[b]	0.4 ± 0.02[a]	0.8 ± 0.01[c]
Total n-6	24.8 ± 0.4[a]	20.9 ± 0.4[b]	12.7 ± 0.4[c]	11.8 ± 0.2[c]
C18:3n-3	0.2 ± 0.02[a]	2.0 ± 0.04[b]	0.2 ± 0.02[a]	0.14 ± 0.01[a]
C20:5n-3	0.2 ± 0.02[a]	0.8 ± 0.03[b]	1.9 ± 0.05[c]	0.9 ± 0.02[a,b]
C22:5n-3	2.6 ± 0.1[a]	5.8 ± 0.1[b]	3.5 ± 0.2[c]	1.9 ± 0.03[d]
C22:6n-3	15.4 ± 0.5[a]	17.4 ± 0.5[a]	24.0 ± 1.7[a]	29.9 ± 0.5[c]
Total n-3	18.4 ± 0.6[a]	26.3 ± 0.4[b]	29.8 ± 1.9[c]	32.8 ± 0.5[c]
MUFA/n-3 ratio	1.15	0.61	0.48	0.34
n-6/n-3 ratio	1.34	0.80	0.43	0.34

Individual fatty acid values are expressed as mean ± SEM (n = 13-15 animals per treatment). [abcd]$P < .05$, means in a row with different superscript letters are significantly different.

trend for lower nonfasting values in the animals consuming the MUFA control and the ALA blend as compared with the animals consuming the marine oil–containing LFD. Significantly higher blood insulin levels were observed in the group consuming EPA blend ($P < .05$); levels peaked after 2 weeks of feeding and continued to be elevated throughout the study. No differences among groups were found in levels of HbA_{1c} at the end of the 4-week feeding period (data not shown).

3.2. Meal tolerance test

The fasting baseline (time 0) plasma glucose values were elevated significantly ($P < .05$) in animals consuming EPA-rich (381 ± 17 mg/dL) and DHA-rich (392 ± 21 mg/dL) but not ALA-rich (348±26 mg/dL) LFD as compared with mice consuming the MUFA control (299 ± 25 mg/dL). The MTT glucose data were then analyzed after adjusting to each animal's fasting glucose value. As shown in Fig. 1, the adjusted plasma glucose response to a standard meal was reduced in all groups that had previously consumed the n-3 PUFA-rich LFD when compared with the group consuming the MUFA control. The plasma glucose response was significantly lower in the animals consuming the EPA and DHA blends at 15 minutes, in the DHA blend group at 120 minutes, and in the group consuming the ALA blend at 60 minutes. However, the 2-hour ΔAUC plasma glucose response was reduced by 40% to 50% in animals that had previously consumed the n-3 PUFA-rich LFD when compared with those animals consuming the MUFA control ($P < .05$).

3.3. Insulin tolerance test

The plasma glucose response to a standard insulin injection was also assessed in a subgroup of animals (n = 5) that did not undergo the MTT (Fig. 2). Only the animals consuming the ALA blend showed a significant and marked reduction in plasma glucose after the insulin injection ($P < .05$ vs MUFA control, and EPA and DHA blends at 30, 60, and 120 minutes and ΔAUC).

3.4. Plasma triglycerides and free fatty acids

As expected, plasma levels of triglycerides and free fatty acids were reduced in the animals consuming the n-3–rich LFD (Fig. 3). Triglycerides were lowered by ~40% ($P < .05$). Free fatty acids were also reduced by ~20% to 25%; however, the reduction was statistically significant ($P < .05$) only in the animals consuming the EPA and DHA blends.

Table 5
Fatty acid composition of thoracic aorta from *ob/ob* mice fed with LFD

Fatty acids (rel %)	Control MUFA blend	ALA blend	EPA blend	DHA blend
C14:0	2.4 ± 0.3[a]	2.3 ± 0.3[a]	3.8 ± 0.2[b]	2.4 ± 0.5[a]
C16:0	23 ± 1.1[a]	24 ± 1.3[a]	29 ± 0.7[b]	29 ± 1.4[b]
C18:0	27 ± 1.8[a,b]	27 ± 0.8[a]	21 ± 1.2[b]	24 ± 2.0[a,b]
Total SFA	53 ± 2.3	54 ± 2.1	55 ± 2.0	56 ± 2.1
C16:1n-7	1.2 ± 0.2[a]	1.3 ± 0.1[a]	3.4 ± 0.3[b]	2.6 ± 0.4[b]
C18:1n-7	2.2 ± 0.1[a]	2.0 ± 0.1[a,b]	2.0 ± 0.1[b]	2.2 ± 0.1[a]
C18:1n-9	26 ± 0.8[a]	23 ± 0.9[b]	22 ± 1.0[b]	23 ± 1.1[b]
C20:1n-9	1 ± 0.1[a]	0.7 ± 0.1[a]	0.5 ± 0.1[b]	0.6 ± 0.1[b]
Total MUFA	31 ± 0.8[a]	28 ± 0.9[b]	29 ± 1.3[b]	29 ± 1.4[a,b]
C18:2n-6	9.4 ± 1.0	10.2 ± 1.0	9.2 ± 0.8	8.4 ± 0.8
C18:3n-6	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.3	0.5 ± 0.1
C20:3n-6	0.4 ± 0.1[a]	0.5 ± 0.1[a]	0.2 ± 0.1[a]	0.04 ± 0.02[b]
C20:4n-6	2.9 ± 0.7[a]	2.5 ± 0.4[a]	0.9 ± 0.1[b]	1.0 ± 0.3[b]
Total n-6	12.9 ± 0.3[a]	13.6 ± 0.3[a]	10.9 ± 0.3[b]	10 ± 0.3[b]
C18:3n-3	0.24 ± 0.1[a]	1.3 ± 0.2[b]	0.3 ± 0.1[a]	0.22 ± 0.1[a]
C20:5n-3	0.14 ± 0.1[a]	0.41 ± 0.1[a]	1.41 ± 0.4[b]	0.47 ± 0.2[a]
C22:5n-3	0.12 ± 0.1[a]	0.56 ± 0.1[b]	0.36 ± 0.1[b,c]	0.16 ± 0.1[a,c]
C22:6n-3	0.77 ± 0.3	1.25 ± 0.3	1.25 ± 0.2	1.83 ± 0.6
Total n-3	1.3 ± 0.5[a]	3.6 ± 0.7[b]	3.5 ± 0.7[b]	2.8 ± 0.8[b]
MUFA/n-3 ratio	23.85	7.78	8.28	10.36
n-6/n-3 ratio	9.92	3.78	3.11	3.57

Individual fatty acid values are expressed as mean ± SEM (n = 14-15 animals per treatment). [abcd]$P < .05$, means in a row with different superscript letters are significantly different.

3.5. Tissue lipid composition

Hepatic total lipid was significantly reduced in mice consuming the EPA blend (198 ± 16 mg/g) and DHA blend (200 ± 16 mg/g), but not in those consuming the ALA blend (270 ± 23 mg/g), compared with the MUFA control group (258 ± 47 mg/g). As expected, the fatty acid profile of the adipose tissue reflected the diet composition (data not shown). The fatty acid composition of the liver, skeletal muscle, and thoracic aorta tissue fractions are shown in Tables 3–5. The liver and skeletal muscle phospholipid fatty acid composition showed similar changes in total saturated fatty acids (SFA), MUFA, and n-6 and n-3 fatty acids. Total SFA was highest in skeletal muscle and hepatic phospholipids, and total MUFA (including C18:1n-9) was lowest in animals fed with the EPA- and DHA-rich diets. Total n-6 fatty acids were decreased in all groups fed with n-3 diets compared with the MUFA control and was the lowest in animals fed with LFD rich in EPA and DHA; this was primarily due to a reduction in C20:3n-6 and C20:4n-6 fatty acids in liver and all major n-6 PUFA in muscle. Mice fed with the ALA blend also showed a reduction in total n-6 PUFA, although unexpectedly, C18:2n-6 was significantly higher in liver phospholipids in animals fed with the ALA-rich diets as compared with EPA and DHA.

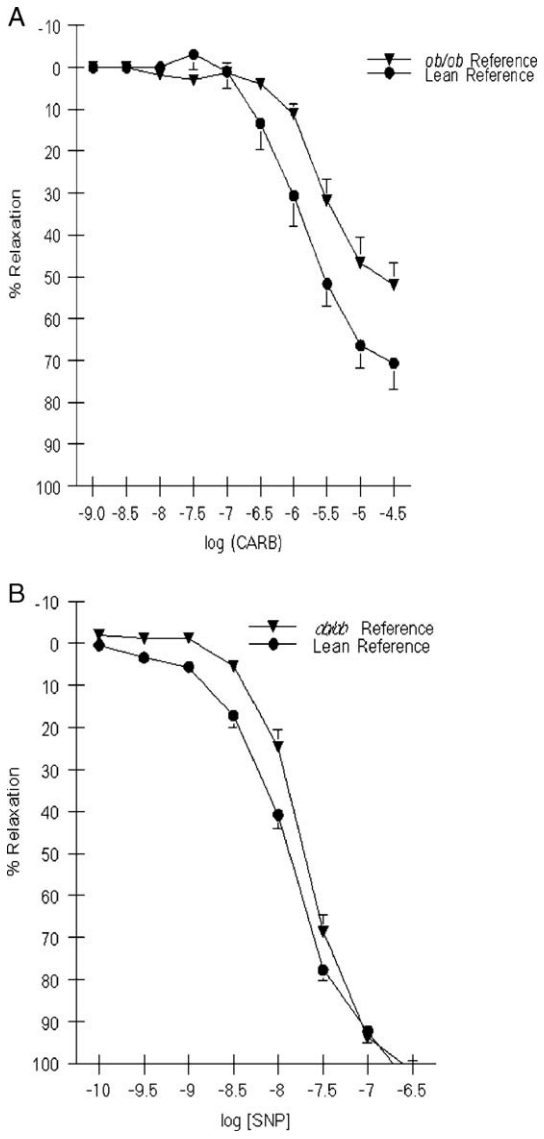

Fig. 4. Vascular reactivity dose-response curves to endothelium- dependent and endothelium-independent vasodilators on PE-contracted aortas from *ob/ob* and lean mice consuming standard chow diets. A, Response to CARB (n = 5 animals per group). $P < .05$ between groups for both pEC_{50} and E_{max}. B, Response to SNP. Not significant between curves.

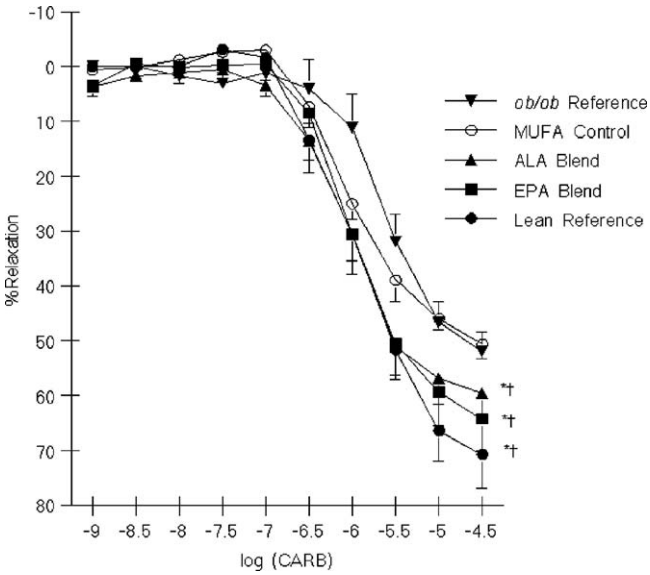

Fig. 5. Dose-response curves to the endothelium-dependent vasodilator, CARB, on PE-contracted aortas from *ob/ob* or lean reference consuming standard chow or *ob/ob* mice consuming LFD for 28 days (n = 5-6 animals per treatment). $P < .05$ for pEC_{50} for all groups compared with *ob/ob* reference. $^{*}P < .05$, E_{max} significantly different vs *ob/ob* reference. $^{\dagger}P < .05$, E_{max} significantly different vs control MUFA.

In contrast, an increase in the proportion of n-3 PUFA was observed in the liver and skeletal muscle phospholipids in animals fed with the n-3–rich diets. Both C18:3n-3 and C20:5n-3, but not C22:6n-3, were increased in tissues in animals fed with the ALA blend. An increase in C20:5n-3 was observed in all animals fed with the n-3 fatty acid–rich diets, with the greatest increase observed in those receiving the preformed C20:5n-3. C22:6n-3 was the predominant n-3 PUFA in liver and skeletal muscle phospholipids in all groups, and increased only in mice consuming preformed DHA in their diets.

A similar pattern was observed in changes in the fatty acid composition of the thoracic aorta (Table 5); however, relative to the other tissues analyzed, the changes were rather modest. SFA C14:0 and C16:0 were elevated in the EPA and DHA groups, although total SFA was not significantly different among groups. Total MUFA including C18:1n-9 was highest in MUFA control group, and total n-6 PUFA including C20:4n-6 was lowest in the groups fed with the marine oil diets. The n-3 fatty acid composition was reflective of the diet composition with C18:3n-3 significantly increased in

the ALA blend group, C20:5n-3 highest in the EPA blend group, and C22:6n-3 highest (not significant) in the DHA blend group.

3.6. Vascular reactivity

Fig. 4A demonstrates the inherent endothelial dysfunction in *ob/ob* mice. The endothelium-dependent relaxations (pEC_{50}) to CARB in the aortic rings of reference *ob/ob* mice on standard chow (5.58 ± 0.1, n = 5) were significantly attenuated (rightward shift, $P < .05$) as compared with those from lean littermates (5.89 ± 0.1, n = 5). Similarly, maximal relaxation (E_{max}) was reduced ($P < .05$) in the *ob/ob* mice (55.2 ± 4.8) as compared with the lean mice (66.7 ± 5.7). Fig. 4B shows the endothelial-independent responses to SNP were not significantly different between *ob/ob* and lean littermates, demonstrating that the endothelial dysfunction does not extend to the vascular smooth muscle.

Fig. 5 shows that long-term feeding of LFD improved pEC_{50} responses (eg, curves showed a leftward shift) after consuming the MUFA control LFD (5.97 ± 0.07, n = 6), ALA blend (6.03 ± 0.1, n = 5), and EPA blend (5.95 ± 0.1, n = 5) when compared with standard chow (5.59 ± 0.08, n = 6). There were no differences in pEC_{50} among LFD groups. There was no improvement in E_{max} in animals consuming the MUFA control as compared with reference *ob/ob* mice consuming standard chow. However, % maximal relaxation of aortas from animals consuming ALA blend (63.7 ± 2.8%) or EPA blend (68.6 ± 4.6%) was greater compared with both the reference *ob/ob* mice and *ob/ob* mice fed with the MUFA control (52.9 ± 3.4%). Aortas from animals consuming n-3 fatty acids were not significantly different compared with lean animals consuming standard chow (66.7 ± 5.7%). Lipid-modified formula diet feeding had no effects on vasorelaxant responses to SNP (not shown).

4. Discussion

This study is the first to demonstrate that long-term consumption of a diabetic nutritional formula containing n-3 PUFA of different chain lengths (C18-C22) and number of double bonds (3, 5, and 6) has differential effects on glucose metabolism, insulin sensitivity, and vascular reactivity in the *ob/ob* mouse model of insulin resistance and T2DM. Mice fed an LFD with EPA and/or DHA blends had lower blood triglycerides and free fatty acids, but insulin sensitivity and fasting plasma glucose were not improved. However, mice fed with the ALA blend had significantly improved insulin sensitivity when compared with those fed with other LFD. Despite these differential effects on the metabolic control, animals fed with LFD rich in n-3 PUFA from either marine or plant sources showed significantly improved vascular responses compared with the MUFA-rich LFD and *ob/ob* reference mice consuming chow.

Most studies in rodents have shown that marine oil n-3 PUFA have a protective effect against high-fat diet-induced insulin resistance [12,14]. Consumption of these fatty acids increases the unsaturation of structural membrane lipids, which enhances insulin action [15]. In addition, marine n-3 PUFA inhibit lipid accumulation and storage in muscle and liver by directing fatty acids away from intracellular storage and toward fatty acid oxidation, improving insulin sensitivity [16,17]. These effects can also be explained at the molecular level by the prevention of many alterations of insulin signaling induced by a high-fat diet [18,19]. Although these effects are important with respect to preventing insulin resistance, however, the results of our study show that supplementation with EPA and/or DHA n-3 PUFA failed to reverse insulin resistance. Similar findings have also been reported in animals [20] or patients with insulin resistance or T2DM [21].

An explanation for the differential effects of the n-3 PUFA on insulin sensitivity and glycemic responses is not clear; however, the results of the present study are supported by those of Podolin et al [20] who concluded that there is a dissociation between n-3 PUFA and lipid metabolism and insulin action in the insulin-resistant state. These data also suggest that the different n-3 PUFA likely have very different effects on hepatic and peripheral tissues. For example, in the current study, the degree of membrane fatty acid unsaturation was increased and hepatic (and plasma) TG was reduced in animals fed with EPA- and DHA-rich diets. These changes, however, were associated with improved meal tolerance glycemic responses, but not with improved insulin sensitivity. In contrast, animals consuming the ALA-rich diet showed marked improvement in insulin resistance, but this was not associated with a decrease in hepatic lipid content. Furthermore, although membrane lipid unsaturation was increased in muscle and hepatic tissues after ALA feeding, these changes were not as marked as compared with those observed in tissues from animals fed with the more highly unsaturated EPA- and DHA-rich diets.

It is possible that specific change(s) in the membrane fatty acid composition may be more important than the overall increase in membrane unsaturation. For example, animals fed with the ALA-rich diet had a smaller reduction in total MUFA and n-6 PUFA and a more modest increase in total n-3 PUFA in liver and muscle when compared with the animals fed with EPA- and DHA-rich diets. It is possible that the combination of MUFA plus the shorter-chain n-3 PUFA in structural membranes allows for more efficient use of insulin and transport of glucose. Previous studies have shown that diets rich in MUFA improve glycemic control compared with low-fat diets [2-5]; thus, it may be important to maintain a critical proportion of MUFA in membrane phospholipids to optimize the efficacy of dietary lipid modification. The importance of both ALA and MUFA has previously been described by Louheranta et al [22]. They showed that the proportions of both of these fatty acids in plasma phospholipids were positively associated with fasting plasma glucose and insulin sensitivity in subjects at baseline and after consuming MUFA-modified diets.

Taken together, these results suggest that the ratio of both ALA and MUFA in the diet may be important for people with insulin resistance and T2DM.

Kato et al [23] showed improvements in both plasma glucose and insulin sensitivity after ALA was provided (as free fatty acid) to hyperglycemic/insulin-resistant KK-Ay mice consuming low-fat chow. These changes coincided with an increase in GLUT4 protein content in skeletal muscle. However, when these mice were fed with ALA in a high-fat/low-MUFA diet, no significant reduction in plasma glucose was observed [24]. Furthermore, studies evaluating the effect of ALA in subjects with T2DM [25,26] or impaired glucose tolerance associated with obesity [27] showed no improvements in variables associated with glucose metabolism or insulin sensitivity. At the present time, a definitive explanation for the inconsistent observations among studies is not obvious. In the latter studies with human subjects, there was little control over the remainder of the diets; thus, an increase in energy expenditure or alteration in other dietary lipids or nutrients may all contribute to the lack of effect on insulin sensitivity. Unfortunately, the MUFA/n-3 ratio in the background diets was not reported.

It is of interest that although only the group on the ALA-rich diet showed improved insulin sensitivity, the postprandial glucose response to a standard meal challenge was also improved in the animals consuming diets rich in marine oil n-3. These results suggest a possible enhancement in meal-stimulated insulin sensitivity and/or insulin secretion. Although the postprandial blood insulin was not measured in the current study, an effect of the marine oil diets, particularly those rich in EPA, on blood insulin is clearly illustrated in the weekly results. The dietary fat composition has been shown to influence glucose-stimulated insulin secretion, with saturated fatty acids being more insulinogenic than unsaturated fatty acids [28]. The marine oil LFD provided more saturated fatty acids compared with the other diets, which could also have contributed to the differential postprandial responses.

Despite the differential effects of n-3 PUFA on glucose metabolism, we observed that both diets improved vascular reactivity responses. Abnormal endothelium-dependent vasodilation is a characteristic of insulin resistance and T2DM as clearly illustrated in the shifts in pEC_{50} and E_{max} in the response curves in the *ob/ob* mice vs the lean littermates. Of interest was that improvements in pEC_{50} were observed in arteries from animals fed with all-liquid formula diets compared with the *ob/ob* chow reference group, whereas E_{max} was also improved in the groups fed with diets containing n-3 PUFA such that the curves resembled those from the nondiabetic reference group consuming chow. Both E_{max} and pEC_{50} are regarded as important indicators of vasoreactivity in isolated aortic rings, although studies in *ob/ob* mice suggest that they likely are affected by different mechanisms [29]. Stimulation of the vascular endothelium with an agonist such as carbachol releases vasodilating substances as well as nitric oxide, and any of these substances can affect the contractility of the vascular smooth muscle. The shift in pEC_{50} may reflect an overall improvement in metabolic responses to the carbohydrate-reduced and nutrient-rich formula diets as compared with the group consuming chow. It may also reflect a specific effect of the MUFA composition as other studies have shown MUFA-rich diets can affect vascular adhesion molecules [30] and improve vascular responses [30-32].

There is considerable evidence supporting the benefits of marine oil n-3 PUFA on vascular function. Both EPA and DHA have been shown to affect vascular tone directly as a result of changes in the fatty acid profile of membrane lipids of endothelial cells as well as through indirect effects on inflammatory cytokines or reduction of atherogenic lipid profile [33,34]. Changes in the fatty acid profile of the thoracic aorta in the *ob/ob* mice would be expected to affect the structural tone as well as production and release of vasodilating substances and nitric oxide. Whether these mechanisms also apply to the observed effects of the ALA-rich LFD is unknown. However, our data in an animal model of insulin resistance and T2DM are consistent with the human study by Nestel et al [27] who demonstrated in obese subjects with glucose intolerance that long-term intake of diets rich in both MUFA or ALA improved arterial compliance compared with diets rich in saturated fatty acids, but more so after the ALA diet than the MUFA-rich diet. In addition, West et al [35] showed that in subjects with T2DM and high triglycerides, the addition of plant or marine oil n-3 PUFA to a MUFA-rich meal improved vascular reactivity up to 4 hours when compared with a MUFA-rich meal containing a very low level of n-3 PUFA. Further work to elucidate these mechanisms is needed.

In summary, the results of the present study show that long-term consumption of diets rich in n-3 PUFAs improved blood lipids and vascular function, but only MUFA diets rich in ALA improved sensitivity to insulin in an animal model with primary characteristics of metabolic syndrome including dyslipidemia, insulin resistance, and T2DM. Further studies of the combination of MUFA- and ALA-rich diabetic formulas in individuals with T2DM are warranted.

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