Original Article
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Obesity

Enhanced Peroxisomal β-Oxidation Is Associated with Prevention of Obesity and Glucose Intolerance by Fish Oil-Enriched Diets

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Objective: The effects of different amounts of omega 3—polyunsaturated fatty acids in diets with normal or high content of fat on lipid and carbohydrate metabolism were investigated.
Design and Methods: Mice were fed for 8 weeks on diets enriched with fish oil or lard at 10% or 60% of energy. Energy balance and energy expenditure were analyzed. Fatty acid (FA) oxidative capacity of the liver and the activity of enzymes involved in this pathway were assessed.
Results: Fish oil-fed mice had lower body weight and adiposity compared with lard-fed animals, despite having lower rates of oxygen consumption. Mice fed diets containing fish oil also displayed lower glycemia, reduced fat content in the liver, and improved glucose tolerance compared with lard-fed animals. The fish oil-containing diets increased markers of hepatic peroxisomal content and increased the generation of metabolites derived from FA β-oxidation in liver homogenates. In contrast, no changes were observed in the content of mitochondrial electron transport chain proteins or carnitine palmitoyl transferase-1 in the liver, indicating little direct effect of fish oil on mitochondrial metabolism.
Conclusion: Collectively, our findings suggest that the energy inefficient oxidation of FAs in peroxisomes may be an important mechanism underlying the protection against obesity and glucose intolerance of fish oil administration.

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Introduction

Saturated fat oversupply is strongly associated with the development of insulin resistance (1). However, the precise mechanisms linking excess lipid availability with impaired insulin action are not fully defined. Defects in mitochondrial function, accumulation of lipid metabolites (e.g., diacylglycerols and ceramides), disruption of insulin signaling, and activation of inflammatory, oxidative, and endoplasmic reticulum stress pathways have been proposed to play a role (2-5).

Although a large amount of literature points to a causative role of lipids in precipitating insulin resistance and metabolic disease, it is clear that not all fatty acid (FA) subtypes induce the same effects. For example, we have recently shown that diets rich in medium chain FA do not induce the same degree of adiposity or insulin resistance as diets containing long-chain saturated and monounsaturated ones (6). Another class of FA that have also been shown to be beneficial for insulin action are omega-3 (n-3) polyunsaturated fatty acids (PUFA), which are found in high concentrations in fish oil. Dietary supplementation with n-3 PUFA has been shown to prevent insulin resistance induced by sucrose or high-fat diets in rodents (7-11) and beneficial effects of n-3 PUFA on insulin action and blood lipid levels have also been reported in humans (12).

Omega-3 PUFA can affect various intracellular pathways that could underlie the beneficial effects of this class of FA on insulin action. Diets enriched with fish oil have been shown to enhance mitochondrial biogenesis and increase secretion of the insulin-sensitizing adipokine, adiponectin (13,14) in adipocytes. Omega-3 PUFA are also known to inhibit lipogenesis and stimulate pathways of FA

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oxidation, the latter being dependent on the interaction of n-3 PUFA with peroxisome proliferator activated receptor alpha (PPARα) (9). More recently, studies have shown that the insulin-sensitizing effects of n-3 PUFA may be partially due to anti-inflammatory effects, mediated by the G-protein coupled receptor GPR120 or the production n-3-derived inflammatory resolution mediators (11,15).

The aim of this study was to investigate the effects of diets enriched with 3.6% or 32% fish oil on lipid and carbohydrate metabolism. The results were compared with those from mice fed lard-containing diets and show that mice fed diets rich in n-3 PUFA, did not accumulate as much adipose tissue and remained more glucose tolerant despite a paradoxical reduction in whole-body energy expenditure.

Methods and Procedures

Animal model

Ten-week-old male Swiss mice were obtained from the Central Animal Facility of the University of Campinas (Campinas, Brazil) or from the Animal Resources Center (Perth, Australia). The animals were kept on a 12-h light/dark cycle with free access to water and food. All experiments were approved by Ethics Committees from the Institute of Biomedical Sciences, the University of São Paulo, and from the Garvan Institute/St. Vincent's Hospital and were carried out following the Brazilian and Australian guidelines for animal research.

Experimental design

Mice were divided into four groups. Each one received different diets for 8 weeks. Two groups were fed diets where fish oil was used as the main lipid source (90% fish oil and 10% soybean oil). One of these groups received a normolipidic diet, where the lipid content was 10% of the total energy (referred to as NFO) and the second group was fed a hyperlipidic diet, where fat content was 60% of the total energy (referred to as HFO). Two more groups were fed on diets prepared using lard as the main lipid source (90% lard and 10% soybean oil). From these groups, one received a normolipidic diet (NL group) and the other a hyperlipidic diet (HL group). The characteristics of the diets, including FA composition determined by gas chromatography, can be found in Table 1.

TABLE 1 Diets composition

Ingredients (g/kg)	NFO	HFO	NL	HL
Starch	621	248	621	248
Sucrose	100	100	100	100
Casein	140	200	140	200
Soybean oil	4	35	4	35
Fish oil	36	315	-	-
Lard	-	-	36	315
Energy (kJ/g)	10.6	22.5	10.6	22.5
Fatty acid composition (% total lipids)				
14:0	7.72		1.11	
16:0	19.43		24.1	
16:1	9.4		1.59	
18:0	3.48		13.75	
18:1	17.36		39.25	
18:2n6	9.24		18.03	
18:3n3	3.08		0.97	
20:4n6	17.68		-	
20:5n3	1.78		-	
22:6n3	10.77		-	
Σ saturated	30.63		38.97	
Σ monounsaturated	26.76		40.84	
Σ n-6 PUFA	26.92		18.03	
Σ n-3 PUFA	15.63		0.97	
n-6/n-3	1.72		18.51	

Main components of the experimental diets and their fatty acid composition.

Determination of energy balance

Energy content of diets and faeces was determined using a bomb calorimeter (Model C5000, Ika, Germany). Food intake and fecal excretion were measured in weeks 1, 3, and 7. Energy absorption was calculated subtracting energy excretion in faeces from energy intake and this data was used to calculate energy efficiency, dividing body weight gain by the accumulated energy absorption.

Insulin and glucose tolerance tests

Insulin tolerance test (ITT) was performed at the 7th week of the treatment, at 2:00 PM, 7 h after food withdrawal at 7:00 AM. Insulin was diluted in 0.9% NaCl solution and intraperitonially injected at the dose of 0.75 U/kg of body weight. Blood samples were obtained from a small cut at the tail tip and glucose concentration measured using a glucometer (Accu-check, Roche). Glucose tolerance test (GTT) was performed under the same conditions described for ITT, during the 8th week of the treatment period, by giving intraperitonially 2 g/kg b.w. of glucose. Plasma samples were collected before the injection of glucose for insulin determination. Insulin levels were determined by radioimmunoassay (Linco Research, St. Charles, MO). Glycemia was monitored through the course of the GTT in the same way as in the ITT.

Indirect calorimetry

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) of individual mice were measured using an eight-chamber indirect calorimeter (Oxymax series, Columbus Instruments, Columbus, OH). The results were obtained after 2-3 h of acclimation to the chamber and data was collected at intervals of 27 min over a 24-h period. Room temperature was kept steady at 22°C and mice had free access to water and food.

In vitro glucose metabolism

Estimation of glucose uptake and incorporation into glycogen was performed in isolated soleus muscle as previously described (16). Briefly, soleus muscles were isolated from the mice, attached to stainless steel clips by their tendons, and preincubated in Krebs-Ringer buffer, pH 7.4, containing 5.6 mmol/L glucose, at 31°C under agitation (100 rpm) for 45 min. After the preincubation period, muscles were transferred to vials containing the same buffer with or without 300 μUI/mL insulin, containing 0.2 μCi D-[U-^{14}C]glucose and 0.2 μCi 2-deoxy-D-[2,6-^{3}H]glucose and incubated for 1 h under the same conditions. After the incubation, samples were digested in 1 mol/l KOH aqueous solution and glycogen was precipitated from a portion of this extract. ^{3}H and ^{14}C counts were determined in aliquots of the digested muscle or precipitated glycogen to determine glucose uptake and glucose incorporation into glycogen.

Lipid measurements

The plasma levels of cholesterol, triacylglycerides, and nonesterified FA (NEFA) were determined using enzymatic assays (Quibasa, Belo

Horizonte, Brazil and Wako Pure Chemicals, Osaka, Japan), following the manufacturers instructions. The triglycerides content of the liver and gastrocnemius muscle was measured using an enzymatic assay, after lipid extraction and solubilization in ethanol. Diacylglycerol content of the liver was determined through thin layer chromatography (TLC), after total lipid extraction and solubilization in hexane. TLC was performed according to the method of Nakamura and Handa (17).

Palmitic acid oxidation

Palmitic acid oxidation was measured in liver and tibialis muscle homogenates following an adaptation of the method described by Kim et al. (18). Tissues were homogenized in 19 volumes of 250 mmol/l sucrose, 10 mmol/l Tris-HCl and 1 mmol/l EDTA, pH 7.4. Fifty microliter of tissue homogenate were incubated with 450 μl reaction mixture (100 mmol/l sucrose, 80 mmol/l KCl, 10 mmol/l Tris-HCl, 5 mmol/l KH_2PO_4, 1 mmol/l $MgCl_2$, 2 mmol/l malate, 2 mmol/l ATP, 1 mmol/l dithiothreitol, 0.2 mmol/l EDTA, 0.3% FA-free BSA, 0.2 mol/l palmitic acid, 0.2 μCi [1-^{14}C]-palmitic acid, 2 mmol/l L-carnitine and 0.05 mmol/l coenzyme A). After 90 min of incubation at 30°C, the reaction was stopped by addition of 100 μl 1 mol/l perchloric acid. CO_2 produced from [1-^{14}C]-palmitic acid was collected in 100 μl of 1 mol/l sodium hydroxide solution. The ^{14}C counts from acid soluble metabolites (ASM) present in the acidified supernatant were also determined.

Enzyme activities measurements

Gastrocnemius muscle and liver samples were homogenized (1:19—wt/vol) in 50 mmol/l Tris-HCl, 1 mmol/l EDTA, and 0.1% Triton X-100, pH 7.2, and subjected to three freeze-thaw cycles. Activities of succinate dehydrogenase (SDH), β-hydroxyacyl CoA dehydrogenase, and medium-chain acyl-CoA dehydrogenase were determined at 30°C as previously described (19,20). The activity of peroxisomal Acyl-CoA oxidase (ACO) was determined as described by Small et al. (21). The reactions were monitored using a Spectra Max 250—microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Western blotting analysis

An aliquot of the liver was homogenized in RIPA buffer (65 mmol/l Tris pH 7.4, 150 mmol/l NaCl, 1% nonidet NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing protease and phosphatase inhibitors (10 μg/ml phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 10 μ/ml leupeptin, 1 mmol/l sodium orthovanadate, and 10 mmol/l sodium fluoride), and solubilized for 1 h at 4°C. Equal amounts of tissue lysates (20 μg protein) were resolved in SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against different subunits of the ETC complexes—complex II FeS subunit, complex III core2 subunit, complex V subunit alfa (Mitosciences, Eugene, OR), peroxisomal membrane protein 70 (PMP-70) (ABCam), phosphoenolpyruvate carboxykinase, and pan 14-3-3 as a loading control. Immunolabeled bands were quantitated by densitometry.

WY-14,643 treatment

To investigate the involvement of the transcription factor PPARα and peroxisomes in the effects observed in the fish oil fed animals, a group of Swiss mice, fed on chow diet was treated with WY-14,643, a known PPARα agonist (22,23). The mice were daily treated by gavage with 10 mg/kg b.w. of WY-14,643 or water as control for 7 days (24). Hepatocytes were isolated at the end of the treatment period, following the method of Berry and Friend (25) and palmitic oxidation was measured in freshly isolated hepatocytes. Hepatocytes (2.5×10^5) were incubated in 1 ml Krebs-Henseleit buffer containing 5 mM glucose, 2% BSA, 0.2 mM palmitic acid, and 0.5 μCi/ml 1-[^{14}C]-palmitic acid. After 15 min, the cell suspension was transferred to a sealed vial containing an opened microtube with 1 M NaOH aqueous solution. The cell suspension was acidified with 100 μl 1 M perchloric acid and after 2 h, the content of the microtube was counted in a be β-counter (Beckman LS6500) to calculate CO_2 production. The cell suspension was centrifuged at 12,000 rpm for 15 min and the radioactivity of an aliquot of the supernatant was counted in a β-counter as for determination of ASM. The measurement of these metabolites indicates partial oxidation of palmitic acid—products from β-oxidation that have not been converted into CO_2.

Statistical analysis

Results are presented as mean ± SE. Depending on the nature of the data, Student's *t*-test or two-way ANOVA with post hoc Bonferroni correction were used to identify statistically significant differences among groups. Differences with $P < 0.05$ were considered statistically significant.

Results

The body weight of mice fed lard-containing diets was higher than that of mice receiving diets supplemented with fish oil (Table 2). This increase in body weight is likely a result of the higher energy efficiency. In fact, mice from the HL group showed the highest energy efficiency and body weight. The mass of the retroperitoneal and subcutaneous fat pads was also elevated in the lard-fed animals. So, changes in adiposity underpinned much of the difference in body weight between groups. Despite the much higher lipid content in the diet, the HFO group displayed similar body weight and fat mass as the NFO group.

To determine if the differences in body composition between groups may be related to changes in energy expenditure, indirect calorimetry measurements were conducted. Both fish-oil fed groups displayed lower VO_2 ($P < 0.05$) than their lard counterparts. The mice fed low-fat diets (regardless if enriched with fish oil or lard) displayed higher RER with typical circadian variation (i.e., higher RER in the dark phase compared with the light phase), whereas animals fed both hyperlipidic diets did not display a marked circadian oscillation. The higher CO_2 production by mice fed NL compared with the high-fat-fed mice is associated to the predominant carbohydrate usage as energy source during the dark phase (Figure 1).

Basal glycemia was lower in both groups of fish oil-fed mice compared with lard-fed animals, whereas the insulinemia was reduced in the HFO group only (Table 2). Although these changes in glycemia and insulinema suggest potential differences in insulin sensitivity, all groups displayed similar insulin tolerance. The rate of glycemia decay during the linear phase of the ITT did not differ between the groups (Figure 2). Corroborating the in vivo observations, 2-deoxy-glucose

TABLE 2 Physiological and biochemical data of the mice

	NFO		HFO		NL		HL	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Endpoint body weigth (g)	42.5	1.25	42.8	1.15	46.1	1.25	49.7	1.29[a,b]
Body weight gain (g)	12.37	1.38	12.51	0.96	15.61	1.71	16.81	2.92[a]
Average food intake (g)	5.55	0.11	3.82	0.16[b]	5.50	0.20	3.39	0.14[b]
Average energy in feces (kJ/day)	5.80	0.12	5.32	0.21	5.79	0.20	5.95	0.52
Energy absorption (kJ/day)	92.51	3.54	83.42	4.63	83.72	3.6	77.85	1.50
Energy efficiency (mg/kJ)	3.04	0.15	2.40	0.24	3.86	0.36	4.28	0.33[a]
Fat mass (g)	7.50	0.93	5.48	0.49	8.20	0.97	9.7	1.21[a]
Retroperitoneal fat pad (g)	0.67	0.03	0.61	0.05	0.98	0.06[a]	1.32	0.15[a,b]
Subcutaneous fat pad (g)	1.38	0.1	1.11	0.12	1.53	0.11	2.1	0.2[a,b]
Liver (g)	4.84	0.27	5.3	0.25	4.99	0.05	4.6	0.27
Glycemia (mM)	8.12	0.36	8.55	0.26	9.94	0.32[a]	10.48	0.38[a]
Insulin (pg/ml)	514	67	208	22[a,b]	493	86	664	117
Plasma triglycerides (mg/dl)	146.8	6.41	71.45	6.51[a,b]	168	19.1	134.59	17.51
Plasma cholesterol (mg/dl)	137.91	6.8	132.83	12.6	203.67	19.7[a]	223.23	17.7[a]
Plasma NEFA (mM)	1.49	0.06	0.96	0.09[a,b]	1.54	0.11	1.62	0.25
Liver triacylglycerides (μmol/g)	7.58	1.84	10.71	3.96	24.94	7.75[a]	25.97	4.67[a]
Liver diacylglycerides (μg/g)	229.42	8.84	262.5	11.90	290.27	15.10[a]	348.10	13.65[a,b]

Values are means ± SEM, $n = 10$. Energy efficiency values were calculated based on food intake and faecal excretion data collected in weeks 1, 3, and 7 of the feeding regime. Data were analyzed by two-way ANOVA followed by Bonferroni test.
[a]Statistical significance between groups fed with different fat type.
[b]Statistical differences between groups fed on diets with different content of fat.

uptake (Figure 2) and glycogen synthesis (data not shown) in isolated soleus muscle were not different among the groups under both basal and insulin-stimulated (300 μUI/ml) conditions. In contrast to the ITT results, a significant effect of both dietary fat type and content was found on GTT. Fish oil-fed mice showed an improved ability to handle the glucose load compared with lard-fed mice (Figure 2). Even-though the amount of glucose administered was calculated considering body weight (implying in a 14% higher dose to the HL group), mice from the HFO group displayed a 24% lower incremental area under the glycemia curve (AUC) compared with HL mice (data not shown). Mice from the HFO group also showed reduced activity of PEPCK in the liver, which suggests specific effects of the HFO diet on glucose metabolism in liver.

Plasma levels of cholesterol, triglycerides, and NEFA were reduced in the group fed a high-fat diet containing fish oil compared with the group fed a high-fat diet containing lard (Table 2). Triacylglycerol content of skeletal muscle was not different between the groups (data not shown), but fish oil induced a decrease in the levels of both triacylglycerols and diacylglycerols in the liver (Table 2). The various diets did not change palmitic acid oxidation in skeletal muscle (Figure 3). In contrast to this, a greater production of ASM from

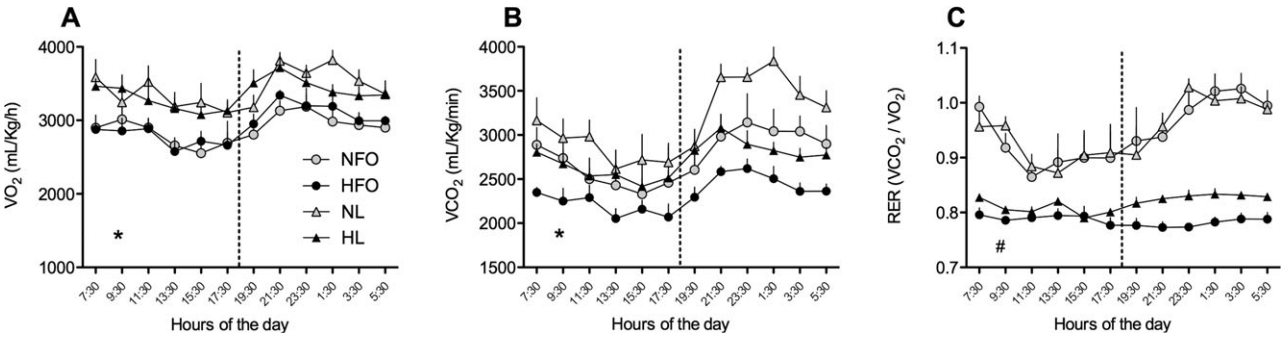

FIGURE 1 Indirect calorimetry. **A**: Oxygen consumption. **B**: CO_2 production. **C**: RER. Gray circles—NFO; black circles—HFO; gray triangles—NL; and black triangles—HL. $n = 5$. *, Differences are statistically significant for the average daily O_2 consumption and CO_2 production between fish oil and lard-fed mice. #, Differences are statistically significant for the average RER between low- and high-fat fed mice. Two-way ANOVA followed by Bonferroni test.

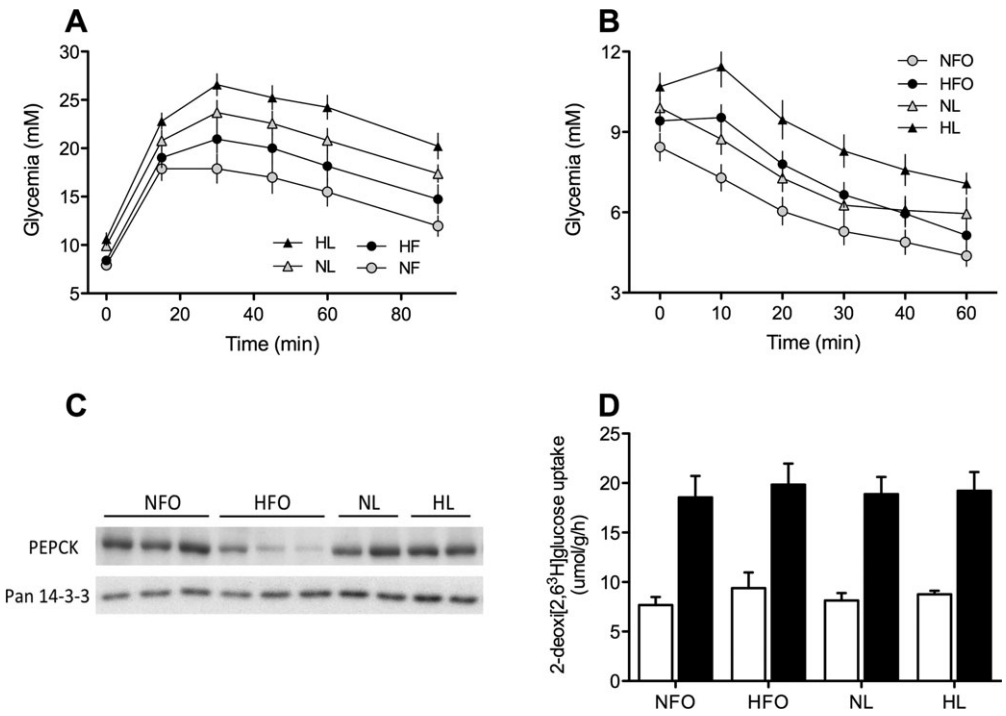

FIGURE 2 Carbohydrate metabolism. **A**: Glucose tolerance test (GTT). **B**: Insulin tolerance test (ITT). During the GTT, Differences are statistically significant for the incremental area under the curve between fish oil and lard-fed mice. No differences detected between groups in the ITT test. Gray circles—NFO; black circles—HFO; gray triangles—NL; and black triangles—HL. **C**: Protein level of PEPCK (and Pan 14-3-3 as a loading control) in liver determined by western blotting. **D**: Isolated soleus muscle 2-deoxy[2,6^{3}H]glucose uptake. White bars—basal; black bars—300 μUI/ml insulin stimulated. Basal 2DG uptake was statistically different from insulin stimulated. No differences observed between groups. $n = 10$, two-way ANOVA followed by Bonferroni's test.

palmitic acid was observed in the liver of fish oil-fed mice, concomitantly with a reduced CO_2 production. The ASM refer to products of FA β-oxidation that were not oxidized to CO_2 and the 2-5-fold higher ASM/CO_2 ratio in fish oil-fed mice (Figure 3) indicates enhanced β-oxidation activity in relation to TCA cycle metabolites flux.

To investigate if the reduction in CO_2 production from palmitate in liver was related to alterations in mitochondrial function, the activities of oxidative enzymes and the levels of mitochondrial respiratory chain proteins were measured. The activity of SDH (data not shown) and the protein content of respiratory chain subunits were not different among the groups (Figure 4). However, the activities of FA β-oxidation enzymes were increased in the fish oil fed groups. In particular, there was a marked increased in the ACO, an enzyme exclusive to the peroxisome. The content of the peroxisomal protein PMP-70 was also substantially increased by the fish oil diets (Figure 4). In line with the palmitate oxidation results, there was little difference in the activity of SDH or β-oxidation enzymes in skeletal muscles of the four groups (data not shown).

To assess the involvement of peroxisomes in the differential metabolism of FA observed the fish oil-fed group (i.e., increased ASM and decreased CO_2), hepatocytes were isolated from mice treated with the PPARα agonist Wy-14,643, and 1-[^{14}C]-palmitic acid oxidation was then assessed. Similar to the findings in liver homogenates from fish oil-fed mice, hepatocytes from Wy-14,643-treated mice produced ~60% less CO_2 from palmitic acid, but generated around ~300% more ASM, when compared with control hepatocytes (Figure 5).

Discussion

Supplementation of n-3 PUFA is known to have beneficial effects on insulin action and blood lipid levels (26-30). However, there is still a gap in our mechanistic understanding of the pathways responsible for these effects. In the current study, we have shown that mice fed either low or high-fat diets enriched with fish oil displayed reduced adiposity and better glucose tolerance, compared with mice fed similar diets enriched with lard. Mice provided with a hyperlipidic diet enriched with n-3 PUFA displayed the most favorable metabolic phenotype with respect to adiposity and circulating lipids and improved glucose tolerance. Intriguingly, despite a similar intake of calories, we observed that the reductions in adiposity induced by fish oil diets could not be explained by enhanced energy expenditure, as both fish oil fed groups exhibited lower whole-body O_2 consumption. This apparent anomaly of reduced fat mass, despite similar food intake and reduced energy expenditure, might be explained by an inability to detect small but cumulatively significant changes in food intake, malabsorption of nutrients or nutrients being metabolized and excreted via a mechanism not involving complete oxidation. Considering that there was no detectable difference in food

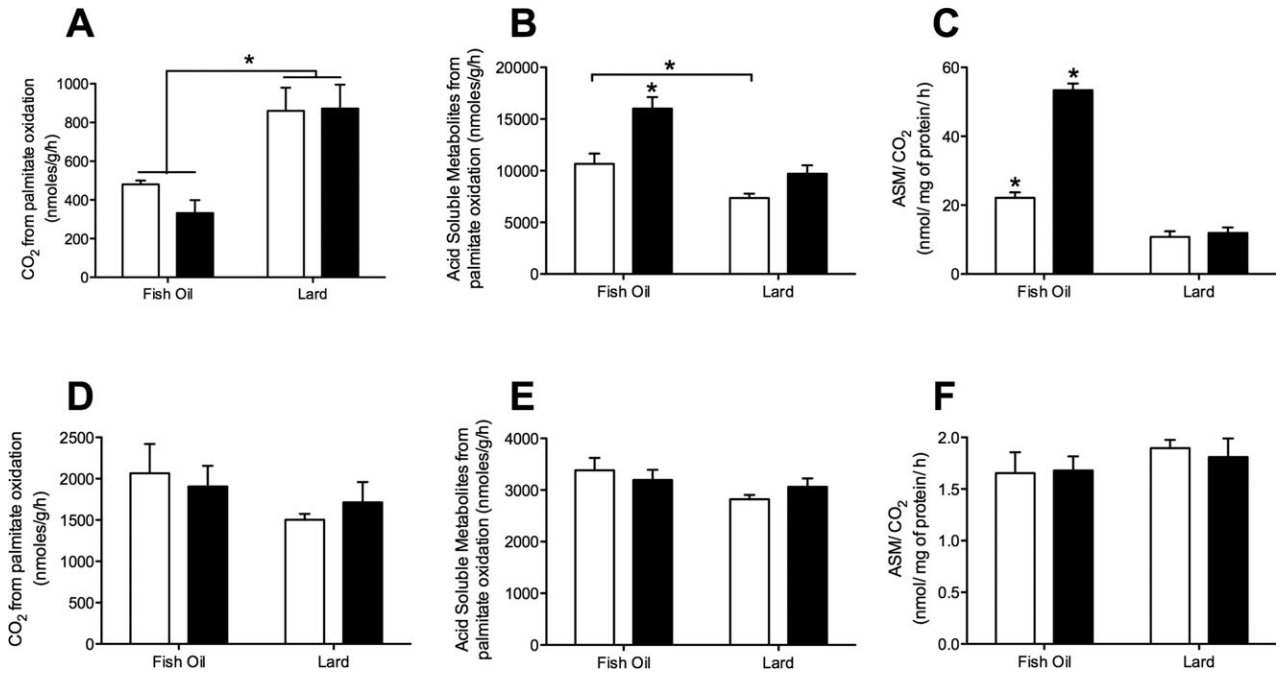

FIGURE 3 1-[^{14}C]-Palmitic acid oxidation in the liver and tibialis muscle homogenates. **A**: CO_2 production from palmitic acid in the liver. **B**: ASM production from palmitic acid in the liver. **C**: Ratio between ASM and CO_2 production in the liver. **D**: CO_2 production from palmitic acid in tibialis muscle. **E**: ASM production from palmitic acid in tibialis muscle. **F**: Ratio between ASM and CO_2 production in muscle. White bars—low-fat diet; black bars—high-fat diets. $n = 5$. $^*P < 0.05$ in relation to the other groups, unless indicated. Two-way ANOVA followed by Bonferroni test.

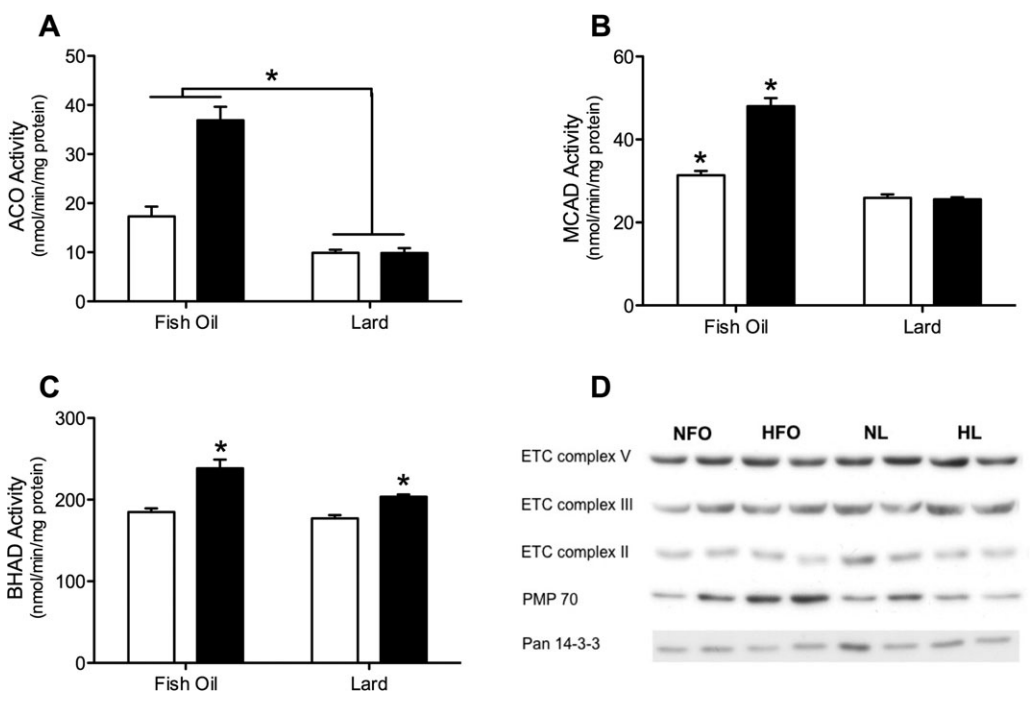

FIGURE 4 Activity of β-oxidation enzymes in the liver. **A**: Acyl-CoA oxydase. **B**: β-Hydroxy acyl CoA dehydrogenase. **C**: Medium chain acyl CoA dehydrogenase. White bars—low-fat diet; black bars—high-fat diets. $n = 5$. $^*P < 0.05$ in relation to the other groups, unless indicated. Two-way ANOVA followed by Bonferroni test. **D**: Content of complex II, III, and V subunits of the electron transport chain and PMP70 determined through western blotting. Pan 14-3-3 was used as loading control. Displayed bands are representative of $n = 5$.

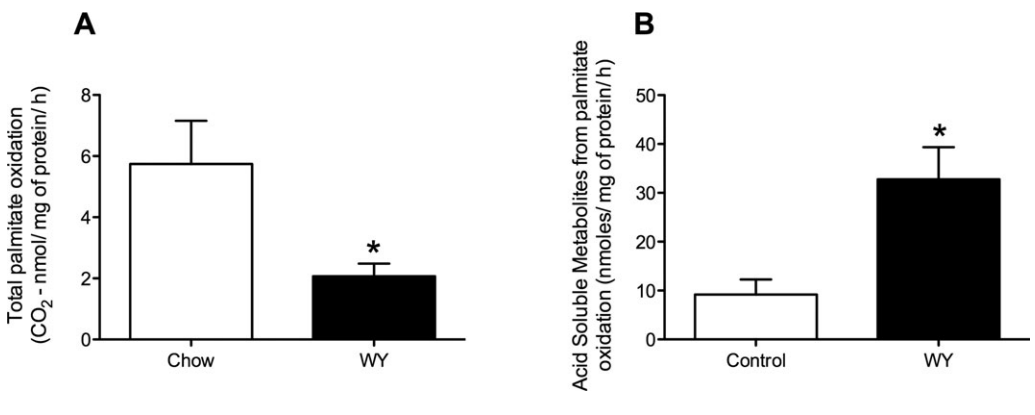

FIGURE 5 1-[^{14}C]-Palmitic acid oxidation in freshly isolated hepatocytes from control or WY-14,643 treated mice. **A**: CO_2 production. **B**: Acid soluble metabolites production. $n = 5$. *$P < 0.05$ as indicated by Student *t*-test.

intake and given that fecal energy content was the same across the groups (Table 2), we will consider the third hypothesis, which will be further discussed.

Both groups of fish oil fed mice displayed reductions in basal glycemia and improved glucose tolerance, whereas the HFO group also exhibited a substantial reduction in circulating insulin levels. The decrease in insulin levels in response to n-3 PUFA has also been reported in another study (31), the mechanism underlying this effect is currently unknown. Overall, the above findings indicate an improvement of insulin action in fish oil fed animals. However, insulin tolerance was similar in all mice and direct assessment of insulin-stimulated glucose uptake in soleus muscles demonstrated no differences across the groups. Aas et al. have shown that EPA does not change insulin-stimulated glucose uptake in cultured human myocytes, although there was an inhibitory effect on glycogen synthesis (32). The HFO group displayed the lowest liver PEPCK activity and this might be associated to the improved response to the GTT. Taking these results together, one can conclude that fish oil had a stronger effect on suppressing hepatic gluconeogenesis than on stimulating glucose uptake in peripheral tissues, leading to the differences observed in the GTT.

Markers of FA oxidative capacity were increased and lipid accumulation was reduced in the liver from fish oil fed mice, whereas only small changes were observed in skeletal muscles. Others and we have previously shown that mitochondrial FA oxidative capacity is increased in muscle of C57BL6 mice in response to high-fat feeding (19,33). So, the lack of induction in the skeletal muscle may be due to differences in mouse strain, as previous work has shown that inbred strains respond differently to high-fat diets (34). Particularly, the Swiss mice are resistant to diet induced obesity. The lack of effect of dietary fish oil on markers of muscle FA oxidative capacity is consistent with the findings of Neschen et al. These authors also observed major effects in the liver but no changes in skeletal muscle (31).

The increased lipid oxidation in the liver of fish oil-fed mice is most likely the result of enhanced peroxisome content (as indicated by the marked increase in the activity of ACO and the PMP70 levels). The increased production of ASM and the reduced CO_2 production from palmitic acid also indicate a preferential channeling of FA into peroxisomes for oxidation. These changes were observed in both the HFO and NFO groups, suggesting that alterations in lipid metabolism occur even at relatively low dose of dietary fish oil. Previous work has reported upregulation of peroxisomal β-oxidation and increased ASM generation from palmitic acid in liver homogenates from fish oil fed mice (35). Other authors reported that the effects of fish oil on FA metabolism in the liver were dependent on PPARα (9). Our findings of increased ASM generation from palmitic acid at the expense of complete mitochondrial oxidation to CO_2 in the liver of fish oil fed mice, and in those treated with the PPARa agonist Wy-14,643 support this idea. The results from palmitic acid oxidation assay suggest that when peroxisome content is increased, more FA can be directed for beta-oxidation in these organelles. Including those that are not normally oxidized there (e.g., palmitic acid).

An enhanced rate of FA oxidation in peroxisomes may potentially explain the observation of reduced body weight gain associated to the lower whole-body O_2 consumption in fish oil-fed mice. This explanation is plausible given that peroxisomes can perform FA β-oxidation using half of the O_2 amount that would be used in mitochondrial β-oxidation. In peroxisomes, during the first step of this pathway, FA oxidation donates electrons to molecular oxygen, generating H_2O_2 which is degraded by catalase, releasing O_2 again. In the mitochondria, FA oxidation donates electrons to the electron transport chain consuming O_2 and contributing to ATP production. So, the first step of peroxisomal β-oxidation does not produce a net reduction of O_2 and does not contribute to ATP production via oxidative phosphorylation as much as the mitochondrial pathway (36,37). Peoples et al. have also reported decreased oxygen consumption during exercise in humans supplemented with n-3 PUFA (38).

We refer to the reduced ability of peroxisomal β-oxidation for generating reducing equivalents (and ATP) per FA molecule used as ''inefficient FA oxidation.'' Inefficient energy conservation through peroxisomal β-oxidation of FA likely contributes to the lower body weight and adiposity of fish-oil fed mice. Mice with high peroxisomal activity need to oxidize more FA molecules to obtain the same amount of energy generated through mitochondrial FA β-oxidation. The fate of acetyl-CoA molecules generated through the

peroxisomal β-oxidation could also plays a role in the lean phenotype of the fish oil-fed mice. Evidence obtained in perfused rat hearts suggests that not all acetyl-CoA generated in the peroxisomes is transferred to the mitochondria (39). Acetyl-CoA derived from peroxisomal β-oxidation may be released as acetate or used to synthesize other complex molecules (37). Thus, the induction of peroxisomal FA oxidation in fish oil-fed animals may lead to the synthesis of compounds that could be ''lost'' or excreted from the body, creating a negative energy balance. Small molecules, like volatile organic compounds, are good candidates to be involved in this mechanism.

In summary, we have shown that fish oil-enriched diets result in enhanced peroxisomal oxidation in the liver, which is energy inefficient and may direct β-oxidation products to pathways that do not lead to energy production or storage. Overall, these effects on peroxisomal FA oxidation could be a major contributor to the protection against obesity and glucose intolerance observed in fish-oil fed mice. O

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References

1. Kraegen E, Cooney G, Turner N. Muscle insulin resistance: a case of fat overconsumption, not mitochondrial dysfunction. *Proc Natl Acad Sci* 2008;105:7627-7628.
2. Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. *Arch Biochem Biophys* 2003;419:101-109.
3. Stratford S, Hoehn K, Liu F, Summers S. Regulation of insulin action by ceramide. *J Biol Chem* 2004;279:36608-36615.
4. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science* 2005;307:384-387.
5. Savage D, Petersen K, Shulman G. Mechanisms of insulin resistance in humans and possible links with inflammation. *Hypertension* 2005;45:828-833.
6. Turner N, Hariharan K, TidAng J, et al. Enhancement of muscle mitochondrial oxidative capacity and alterations in insulin action are lipid species dependent: potent tissue-specific effects of medium-chain fatty acids. *Diabetes* 2009;58:2547-2554.
7. Storlien LH, Kraegen EW, Chisholm DJ, Ford GL, Bruce DG, Pascoe WS. Fish oil prevents insulin resistance induced by high-fat feeding in rats. *Science* 1987;237:885-888.
8. Ghafoorunissa, Ibrahim A, Rajkumar L, Acharya V. Dietary (n-3) long chain polyunsaturated fatty acids prevent sucrose-induced insulin resistance in rats. *J Nutr* 2005;135:2634-2638.
9. Neschen S, Morino K, Dong J, et al. n-3 Fatty acids preserve insulin sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner. *Diabetes* 2007;56:1034-1041.
10. Rossi AS, Lombardo YB, Chicco AG. Lipogenic enzyme activities and glucose uptake in fat tissue of dyslipemic, insulin-resistant rats: effects of fish oil. *Nutrition* 2010;26:209-217.
11. Oh DY, Talukdar S, Bae EJ, et al. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* 2010;142:687-698.
12. Fedor D, Kelley DS. Prevention of insulin resistance by n-3 polyunsaturated fatty acids. *Curr Opin Clin Nutr Metab Care* 2009;12:138-146.
13. Flachs P, Mohamed-Ali V, Horakova O, et al. Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet. *Diabetologia* 2006;49:394-397.
14. Neschen S, Morino K, Rossbacher J, et al. Fish oil regulates adiponectin secretion by a peroxisome proliferator-activated receptor-γ-dependent mechanism in mice. *Diabetes* 2006;55:924–928.
15. White PJ, Arita M, Taguchi R, Kang JX, Marette A. Transgenic restoration of long-chain n-3 fatty acids in insulin target tissues improves resolution capacity and alleviates obesity-linked inflammation and insulin resistance in high-fat-fed mice. *Diabetes* 2010;59:3066-3073.
16. Cuendet GS, Loten EG, Jeanrenaud B, Renold AE. Decreased basal, noninsulin-stimulated glucose uptake and metabolism by skeletal soleus muscle isolated from obese-hyperglycemic (ob/ob) mice. *J Clin Invest* 1976;58:1078-1088.
17. Nakamura K, Handa S. Coomassie brilliant blue staining of lipids on thin-layer plates. *Anal Biochem* 1984;142:406-410.
18. Kim JY, Hickner RC, Cortright RL, Dohm GL, Houmard JA. Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab* 2000;279:E1039-E1044.
19. Turner N, Bruce C, Beale S, et al. Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle. *Diabetes* 2007;56:2085–2092.
20. MacArthur DG, Seto JT, Chan S, et al. An Actn3 knockout mouse provides mechanistic insights into the association between alpha-actinin-3 deficiency and human athletic performance. *Hum Mol Genet* 2008;17:1076-1086.
21. Small GM, Burdett K, Connock MJ. A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase. *Biochem J* 1985;227:205-210.
22. Reddy JK, Mannaerts GP. Peroxisomal lipid metabolism. *Annu Rev Nutr* 1994;14:343-370.
23. Pan D, Mater M, Thelen A, Peters J. Evidence against the peroxisome proliferator-activated receptor α (PPARα) as the mediator for polyunsaturated fatty acid suppression of hepatic L-pyruvate kinase gene transcription. *J Lipid Res* 2000;41:742–751.
24. Frederiksen K, Wulff E, Sauerberg P, Mogensen J, Jeppesen L, Fleckner J. Prediction of PPAR-{alpha} ligand-mediated physiological changes using gene expression profiles. *J Lipid Res* 2004;45:592-601.
25. Berry MN, Friend DS. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol* 1969;43:506-520.
26. Dyerberg J, Bang HO, Hjorne N. Fatty acid composition of the plasma lipids in Greenland Eskimos. *Am J Clin Nutr* 1975;28:958-966.
27. Chambrier C, Bastard J-P, Rieusset J, et al. Eicosapentaenoic acid induces mRNA expression of peroxisome proliferator-activated receptor gamma. *Obes Res* 2002;10:518-525.
28. Nakatani T, Kim H-J, Kaburagi Y, Yasuda K, Ezaki O. A low fish oil inhibits SREBP-1 proteolytic cascade, while a high-fish-oil feeding decreases SREBP-1 mRNA in mice liver: relationship to anti-obesity. *J Lipid Res* 2003;44:369-379.
29. Aguilera AA, Díaz GH, Barcelata ML, Guerrero OA, Ros RMO. Effects of fish oil on hypertension, plasma lipids, and tumor necrosis factor-alpha in rats with sucrose-induced metabolic syndrome. *J Nutr Biochem* 2004;15:350-357.
30. Takeuchi H, Kojima K, Sekine S, Murano Y, Aoyama T. Effect of dietary n-6/n-3 ratio on liver n-6/n-3 ratio and peroxisomal beta-oxidation activity in rats. *J Oleo Sci* 2008;57:649-657.
31. Neschen S, Moore I, Regittnig W, et al. Contrasting effects of fish oil and safflower oil on hepatic peroxisomal and tissue lipid content. *Am J Physiol Endocrinol Metab* 2002;282:E395-E401.
32. Aas V, Rokling-Andersen MH, Kase ET, Thoresen GH, Rustan AC. Eicosapentaenoic acid (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells. *J Lipid Res* 2006;47:366-374.
33. Hancock CR, Han D-H, Chen M, et al. High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proc Natl Acad Sci USA* 2008;105:7815-7820.
34. West DB, Boozer CN, Moody DL, Atkinson RL. Dietary obesity in nine inbred mouse strains. *Am J Physiol* 1992;262 (Part 2):R1025-R1032.
35. Willumsen N, Hexeberg S, Skorve J, Lundquist M, Berge R. Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats. *J Lipid Res* 1993;34:13-22.
36. Wanders R, Vreken P, Ferdinandusse S, et al. Peroxisomal fatty acid α-and β-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochem Soc Trans* 2001;29:250-267.
37. Wanders RJA, Waterham HR. Biochemistry of mammalian peroxisomes revisited. *Annu Rev Biochem* 2006;75:295-332.
38. Peoples GE, McLennan PL, Howe PRC, Groeller H. Fish oil reduces heart rate and oxygen consumption during exercise. *J Cardiovasc Pharmacol* 2008;52:540-547.
39. Bian F, Kasumov T, Thomas KR, et al. Peroxisomal and mitochondrial oxidation of fatty acids in the heart, assessed from the 13C labeling of malonyl-CoA and the acetyl moiety of citrate. *J Biol Chem* 2005;280:9265-9271.