

# n-3 Fatty Acids Preserve Insulin Sensitivity In Vivo in a Peroxisome Proliferator-Activated Receptor- $\alpha$ -Dependent Manner

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Recent studies have suggested that n-3 fatty acids, abundant in fish oil, protect against high-fat diet-induced insulin resistance through peroxisome proliferator-activated receptor (PPAR)- $\alpha$  activation and a subsequent decrease in intracellular lipid abundance. To directly test this hypothesis, we fed PPAR- $\alpha$  null and wild-type mice for 2 weeks with isocaloric high-fat diets containing 27% fat from either safflower oil or safflower oil with an 8% fish oil replacement (fish oil diet). In both genotypes the safflower oil diet blunted insulin-mediated suppression of hepatic glucose production ( $P < 0.02$  vs. genotype control) and PEPCK gene expression. Feeding wild-type mice a fish oil diet restored hepatic insulin sensitivity (hepatic glucose production [HGP],  $P < 0.002$  vs. wild-type mice fed safflower oil), whereas in contrast, in PPAR- $\alpha$  null mice failed to counteract hepatic insulin resistance (HGP,  $P = NS$  vs. PPAR- $\alpha$  null safflower oil-fed mice). In PPAR- $\alpha$  null mice fed the fish oil diet, safflower oil plus fish oil, hepatic insulin resistance was dissociated from increases in hepatic triacylglycerol and acyl-CoA but accompanied by a more than threefold increase in hepatic diacylglycerol concentration ( $P < 0.0001$  vs. genotype control). These data support the hypothesis that n-3 fatty acids protect from high-fat diet-induced hepatic insulin resistance in a PPAR- $\alpha$ - and diacylglycerol-dependent manner. *Diabetes* 56:1034–1041, 2007

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2-[<sup>14</sup>C]DG, 2-deoxy-D-[1-<sup>14</sup>C]glucose; HGP, hepatic glucose production; LC, liquid chromatography; MS, mass spectrometry; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element binding protein.

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Obesity and insulin resistance are strongly linked to perturbations in lipid metabolism (1–3). In rodents, several studies have demonstrated that an overly rich dietary fat content increases intracellular lipid accumulation and causes insulin resistance (4–8). In contrast, when rats are fed a fish oil instead of a safflower oil diet, they are protected from fat-induced insulin resistance (4,9–10). The potential of fish oil preserving insulin sensitivity is likely mediated via polyunsaturated  $\omega$ -3 (n-3) fatty acids, such as DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid). Fish oil affects extracellular and intracellular lipid metabolism and decreases the systemic abundance of lipid metabolites in part by accelerating fatty acid catabolism in insulin-sensitive tissues (4,5,9,11–15). Hence, the specific molecular events underlying fish oil and n-3 fatty acid action with respect to restoration of insulin sensitivity appear to involve peroxisome proliferator-activated receptor (PPAR)- $\alpha$ .

The natural PPAR- $\alpha$  ligands DHA and EPA within physiological concentrations bind to PPAR- $\alpha$  and trigger the following: peroxisome proliferation and transcriptional activation of PPAR- $\alpha$ -responsive genes, such as liver fatty acid binding protein, long-chain fatty acyl-CoA synthetase, peroxisomal acyl-CoA oxidase, bifunctional enzyme, 3-ketoacyl-CoA thiolase, and microsomal 4-hydroxylases CYP4A1 and CYP4A6, among others (5,14,16–19). The transcriptional induction of PPAR- $\alpha$ -responsive genes promotes hepatic intracellular fatty acid uptake, the conversion of fatty acids to their respective fatty acyl-CoA derivatives, and their channeling toward mitochondrial/peroxisomal and endosomal  $\beta$ - and  $\omega$ -oxidation (19). PPAR- $\alpha$  also plays an essential role in extracellular lipid homeostasis, mediating the transcriptional regulation of major high-density and very-low-density apolipoproteins, leading to accelerated lipolysis and clearance of remnant particles caused by changes in lipoprotein lipase and apolipoprotein C-III levels (20,21). In addition, PPAR- $\alpha$  appears to mediate the lipopenic action of leptin in liver and adipose tissue (10).

Because PPAR- $\alpha$  is predominantly expressed in liver (22), we hypothesized that hepatic fish oil action would require functional PPAR- $\alpha$  to transmit its insulin-sensitizing potential via decreasing systemic and hepatic lipid abundance. To test this hypothesis in vivo, PPAR- $\alpha$  null and wild-type mice were fed either a control diet or

isocaloric high-fat diets containing safflower oil with or without an 8% fish oil replacement. Then, 2 weeks later, euglycemic-hyperinsulinemic clamp experiments were performed. The data demonstrate that an 8% fish oil supplement in a high-fat diet partially preserved hepatic insulin sensitivity, and its action depended on functional PPAR- $\alpha$ .

## RESEARCH DESIGN AND METHODS

Mice lacking functional PPAR- $\alpha$  (PPAR- $\alpha$  null), generated by targeted gene disruption of the putative ligand-binding domain coding region (23), and wild-type mice on a Sv129 (Jae substrain) background were purchased from Jackson Laboratory and single-housed under standard vivarium conditions. At 10–12 weeks of age ( $\sim 25$  g body wt), male PPAR- $\alpha$  null (control  $n = 19$ , safflower oil-fed  $n = 27$ , and fish oil-fed  $n = 39$ ) and wild-type mice ( $n = 23$ , 22, and 24) were started on either a standard laboratory diet (7% fat-derived calories, 3.76 kcal/g, control) or isocaloric high-fat diets (59% fat-derived calories, 5.18 kcal/g) containing 27% safflower oil (wt/wt; safflower oil: 78% C18:2n-6) without or with an 8% menhaden fish oil replacement (wt/wt; menhaden fish oil: 16% C20:5n-3, 9% C22:6n-3) and fed for 2 weeks. To restrict hepatic triacylglycerol deposition, one batch of PPAR- $\alpha$  null mice was fed the safflower oil diet for only 1 week ( $n = 6$ ). Diets (control: no. 110700; safflower oil: no. 112245; and fish oil no. 112246, 27% wt/wt fish oil diet used for preparation of 8% fish oil diet) supplemented with minerals and vitamins (nos. 210025 and 310025) were purchased from Dyets and fed ad libitum. Mice received fresh diet every 3rd day, and food consumption rates, body weight gains, whole-body fat gains (MQ10 analyzer; Bruker Optics, Billerica, MA), and rectal body temperatures (BAT-12R; Physitemp Instruments, Clifton, NJ) were monitored. All procedures were approved by the Yale University animal care and use committee.

**Euglycemic-hyperinsulinemic glucose clamp experiments.** Before dietary interventions, mice were divided into seven groups (PPAR- $\alpha$  null: control  $n = 10$ , safflower oil  $n = 7$ , and safflower oil 1 week  $n = 6$ ; fish oil  $n = 8$ ; and wild-type: control  $n = 10$ , safflower oil  $n = 9$ , and fish oil  $n = 10$ ) to match their body weight at the onset of in vivo experiments (Table 2). Intravenous catheters were inserted in the left jugular vein under surgical anesthesia on day 8 or 9 of dietary treatment or day 2 when the safflower oil diet was fed for 1 week only. To prevent hypothermia PPAR- $\alpha$  null mice were kept warm during the first postoperative night. All mice regained preoperative body weights during the 5 to 6 days after surgery. On the day of the experiment, conscious mice were placed in restraining tubes and their tails secured with tape. As previously described (6), during the 120-min basal period, a primed-continuous [ $^3$ -H]glucose infusion (10  $\mu$ Ci bolus, 0.1  $\mu$ Ci/min) was administered to determine whole-body glucose turnover rates. The euglycemic-hyperinsulinemic clamp was initiated by a 3-min insulin prime (Humulin; Eli Lilly, Indianapolis, IN), and then insulin infusion was continued at a constant rate (2.5  $\text{mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ), raising plasma insulin concentrations within a physiological range ( $45 \pm 2$   $\mu\text{U/ml}$ ). Steady-state conditions for plasma glucose concentration ( $133 \pm 3$  mg/dl) and specific activity were achieved within 70 min, and a single 2-deoxy-D-[1- $^{14}$ C]glucose (2-[ $^{14}$ C]DG) bolus was injected at 75 min. Blood samples were collected at 80, 85, 90, 100, 110, and 120 min of the clamp to determine plasma [ $^3$ -H]glucose,  $^3\text{H}_2\text{O}$ , and 2-[ $^{14}$ C]DG specific activities and, for measurement of basal [ $^3$ -H]glucose specific activity, in the final 10 min of the basal period. A plasma sample was obtained during the final 10 min of the basal period for determination of basal insulin levels and, for steady-state insulin levels, at 120 min of the clamp. All infusions were performed using microdialysis pumps (CMA, North Chelmsford, MA), and radioisotopes were purchased from Perkin Elmer Life Sciences and American Radiolabeled Chemicals. At the end of the experiment, animals were anesthetized with intravenous ketamine/xylazine, and liver, gastrocnemius muscle (including soleus muscle), quadriceps muscle, epididymal white adipose tissue, and heart were immediately freeze-clamped and stored at  $-80^\circ\text{C}$  until further analysis. The rate of whole-body glucose turnover was calculated as the ratio of the [ $^3$ -H]glucose infusion rate and plasma [ $^3$ -H]glucose specific activity during steady state. Hepatic [ $^3$ -H]glucose production (HGP) was determined by subtracting the steady-state glucose infusion rate from the rate of whole-body glucose turnover (rate of disappearance, or  $R_d$ ).

**Assays from plasma.** Plasma glucose concentrations were determined with a glucose analyzer (Beckman, Fullerton, CA); plasma immunoreactive insulin, leptin, and adiponectin concentrations via radioimmunoassay (Linco, St. Charles, MO); and plasma triacylglycerol, nonesterified fatty acids, and  $\beta$ -hydroxybutyrate concentrations via reagent kits (nos. 336 and 310-3; Sigma, St. Louis, MO; and NEFA-C; Wako, Richmond, VA). Plasma [ $^3$ -H]glucose, 2-[ $^{14}$ C]DG, and  $^3\text{H}_2\text{O}$  radioactivity were determined in deproteinized plasma samples (Somogyi filtrates) before and after  $^3\text{H}_2\text{O}$  was completely evaporated.

$^3\text{H}$  and  $^{14}\text{C}$  radioactivity were assessed with a liquid scintillation counter (Packard, Meriden, CT).

**Tissue 2-[ $^{14}$ C]DG uptake.** 2-[ $^{14}$ C]DG injected during steady-state conditions of euglycemic-hyperinsulinemic clamp experiments resulted in intracellular accumulation of 2-[ $^{14}$ C]DG-6-phosphate, which was separated from 2-[ $^{14}$ C]DG using ion-exchange columns (Poly-Prep 731-6211; Bio-Rad, Hercules, CA). Tissue 2-[ $^{14}$ C]DG uptake was calculated from the plasma 2-[ $^{14}$ C]DG area under the curve at 80, 85, 90, 100, 110, and 120 min and tissue 2-[ $^{14}$ C]DG-6-phosphate content, as described previously (6).

**Tissue acyl-CoA content.** The extraction procedure was adapted from methods described previously (5,6). We homogenized  $\sim 100$  mg tissue with C17:0 acyl-CoA ester internal standard. After purification (oligonucleotide purification cartridges; Applied Biosystems, Foster City, CA) and a drying step, medium, long-chain, and very-long-chain acyl-CoA fractions were dissolved in methanol/ $\text{H}_2\text{O}$  (1:1 vol/vol) and subjected to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis. A turbo ionspray source was interfaced with an API 3000 tandem mass spectrometer (Applied Biosystems) in conjunction with two 200 Series micropumps and a 200 Series autosampler (Perkin Elmer, Shelton, CT). Total acyl-CoA concentrations were expressed as the sum of individual species.

**Tissue diacylglycerol content.** The extraction procedure was adapted from methods previously described (6). We homogenized  $\sim 100$  mg tissue in ice-cold chloroform/methanol (2:1 vol/vol) containing 0.01% butylated hydroxytoluene and the internal standards 1,3-dipentadecanoin and 1,2,3-trihexadecanoin. Organic and aqueous phase were separated adding chloroform and  $\text{H}_2\text{O}$ , and the organic layer was dried under nitrogen flow and reconstituted with hexane/methylene chloride/ether (95/4.5/0.5 vol/vol/vol). Diacylglycerol was separated from triacylglycerol using preconditioned columns (Sep-Pak Cartridge WAT020845; Waters, Milford, MA), eluted with hexane/ethyl acetate (85/15 vol/vol) under low negative pressure and subjected to LC/MS/MS analysis. Total diacylglycerol content was expressed as the sum of individual species.

**Tissue triacylglycerol content.** The extraction procedure was adapted from methods described previously (5,6). After evaporation of the organic solvent, triacylglycerol concentration (triplicates) was measured with an enzymatic method (Sigma).

**Quantitative RT-PCR-based gene expression analysis.** RNA was isolated from postclamp or basal (6-h fasted mice) tissues (RNeasy kit; Qiagen, Valencia, CA) in combination with DNase digest treatment. After 1.5  $\mu\text{g}$  of total RNA was reverse-transcribed (Stratagene, La Jolla, CA) with an oligo-prime, PCR was performed with a DNA Engine Opticon 2 system (MJ Research, Waltham, MA) using a SYBR green QPCR dye kit (Stratagene). The following primers were used: PEPCK: 5' ACC TCC TGG AAG AAC AAG GA 3' (forward) and 5' CTC ATG GCT GCT CCT ACA AA 3' (reverse); 18S rRNA: 5' TTC CGA TAA CGA ACG AGA CTC T 3' (forward) and 5' TGG CTG AAC GCC ACT TGT C 3' (reverse). Product specificity was verified by running products on an agarose gel. mRNA levels ( $\Delta C_T$  values), normalized to 18S rRNA, were expressed using the comparative method. The 18S rRNA levels showed no statistical difference between genotypes.

**Statistical analysis.** All data are expressed as the means  $\pm$  SE. As appropriate, ANOVA, Fisher post hoc analysis, or two-tailed Student's  $t$  tests were performed. To determine dietary effects, safflower oil and fish oil groups were compared with their respective genotype control group. Genotypic differences between wild-type and PPAR- $\alpha$  null mice were assessed exclusively under control diet conditions.

## RESULTS

**Metabolic phenotype of PPAR- $\alpha$  null mice on control diet.** When fed a control diet for 2 weeks, calorie intakes, body weight gains, whole-body fat gains, and plasma leptin concentrations were comparable in PPAR- $\alpha$  null and wild-type mice (Table 1). Paralleled by increased plasma insulin concentrations, 6-h fasting plasma glucose concentrations were markedly lower at comparable HGP rates in PPAR- $\alpha$  null versus wild-type mice (Tables 1 and 2). Despite similar concentrations of plasma fatty acids, 6-h food-deprived PPAR- $\alpha$  null mice displayed markedly lower plasma  $\beta$ -hydroxybutyrate concentrations and hypertriglyceridemia (Table 1).

**Metabolic phenotype of PPAR- $\alpha$  null mice on isocaloric safflower oil and fish oil diets.** When fed with a safflower oil diet, whole-body fat content and plasma leptin concentrations increased to the same extent in

TABLE 1  
Metabolic and plasma parameters in 6-h fasting PPAR- $\alpha$  null and wild-type mice

	Wild type			PPAR- $\alpha$ null		
	Control	Safflower oil	Fish oil	Control	Safflower oil	Fish oil
<b>Plasma metabolites</b>						
Glucose (mmol/l)	8.0 $\pm$ 0.3	9.3 $\pm$ 0.4*	9.1 $\pm$ 0.3	6.8 $\pm$ 0.5†	9.0 $\pm$ 0.5‡	8.0 $\pm$ 0.4*§
Triacylglycerol (mmol/l)	0.52 $\pm$ 0.04	0.47 $\pm$ 0.05	0.40 $\pm$ 0.06	0.67 $\pm$ 0.05†	0.58 $\pm$ 0.05	0.46 $\pm$ 0.03‡§
Nonesterified fatty acid (mmol/l)	0.46 $\pm$ 0.06	0.46 $\pm$ 0.08	0.44 $\pm$ 0.05	0.48 $\pm$ 0.04	0.40 $\pm$ 0.03	0.40 $\pm$ 0.06
$\beta$ -Hydroxybutyrate (mg/dl)	7.6 $\pm$ 1.7	8.8 $\pm$ 1.3	7.0 $\pm$ 1.5	2.4 $\pm$ 0.3	4.0 $\pm$ 0.2	3.5 $\pm$ 0.3
<b>Plasma hormones and adipocyte cytokines</b>						
Insulin ( $\mu$ U/ml)	16.7 $\pm$ 1.8	24.5 $\pm$ 5.2*	21.8 $\pm$ 2.3	30.6 $\pm$ 2.7	30.0 $\pm$ 3.0	25.7 $\pm$ 1.8
Leptin (ng/ml)	9.5 $\pm$ 0.9	13.1 $\pm$ 1.2	14.3 $\pm$ 1.0*	14.5 $\pm$ 1.5†	22.2 $\pm$ 2.4‡	21.9 $\pm$ 1.6‡
Adiponectin ( $\mu$ g/ml)	7.7 $\pm$ 0.4	7.7 $\pm$ 0.6	11.9 $\pm$ 0.7¶#	6.4 $\pm$ 0.3	7.88 $\pm$ 0.8	31.0 $\pm$ 2.8¶**
<b>Metabolic parameters</b>						
Calorie intake (kcal $\cdot$ g body wt <sup>-1</sup> $\cdot$ day <sup>-1</sup> )	0.48 $\pm$ 0.01	0.52 $\pm$ 0.01*	0.53 $\pm$ 0.01*	0.52 $\pm$ 0.01	0.55 $\pm$ 0.01*	0.54 $\pm$ 0.01
Initial body weight (g)	24.5 $\pm$ 0.4	24.4 $\pm$ 0.4	24.0 $\pm$ 0.3	24.3 $\pm$ 0.5	23.3 $\pm$ 0.4	23.6 $\pm$ 0.4
Body weight gain (g/day)	0.24 $\pm$ 0.02	0.29 $\pm$ 0.03	0.27 $\pm$ 0.03	0.26 $\pm$ 0.03	0.39 $\pm$ 0.04*	0.35 $\pm$ 0.03
Initial whole-body fat content (% body wt)	13.6 $\pm$ 1.1	12.4 $\pm$ 1.3	13.7 $\pm$ 1.6	14.4 $\pm$ 0.9	15.4 $\pm$ 1.0	15.8 $\pm$ 0.9
Whole-body fat gain (% body wt day 0–12)	7.6 $\pm$ 0.5	11.0 $\pm$ 0.7‡	11.2 $\pm$ 1.3‡	8.3 $\pm$ 1.0	15.4 $\pm$ 0.7¶	13.5 $\pm$ 0.7¶
Rectal body temperature ( $^{\circ}$ C)	36.1 $\pm$ 0.2	36.3 $\pm$ 0.3	36.6 $\pm$ 0.3	36.2 $\pm$ 0.2	36.5 $\pm$ 0.2	35.3 $\pm$ 0.6

Data are the means  $\pm$  SE of 14–31 mice per group or 6–9 for body fat gain and rectal body temperature. In male PPAR- $\alpha$  null and wild-type mice, metabolic parameters were obtained during and plasma parameters at the end of 2 weeks' dietary treatment with either control diet or isocaloric high-fat diets containing 27% safflower oil without or with an 8% fish oil replacement. Plasma parameters and rectal body temperatures were determined in 6-h food-deprived mice. \* $P$  < 0.05 vs. genotype control; † $P$  < 0.05 vs. wild-type control; ‡ $P$  < 0.005 vs. genotype control; § $P$  < 0.05 vs. genotype safflower; || $P$  < 0.005 vs. wild-type control; ¶ $P$  < 0.0001 vs. genotype control; # $P$  < 0.005 vs. genotype safflower; \*\* $P$  < 0.0001 vs. genotype safflower.

PPAR- $\alpha$  null and wild-type mice compared with the respective genotype fed control diet (Table 1). Safflower oil feeding was accompanied by 6-h fasting hyperglycemia in both genotypes and by an increase in plasma insulin concentrations solely in wild-type mice (Table 1). When PPAR- $\alpha$  null mice were started on a 27% fish oil diet, they reduced food intake, and their survival rate declined by day 5, reaching 0% by day 13 ( $n$  = 13) (data not shown). In contrast, PPAR- $\alpha$  null mice displayed stable food consumption rates, progressive body weight, and total body fat gains throughout the 2 weeks of dietary intervention on an 8% fish oil diet, and therefore this lower dietary fish oil dose was chosen for all subsequent experiments (Table 1). Feeding mice of both genotypes with the 8% fish oil diet increased their

whole-body fat content and plasma leptin concentrations to a similar extent within 2 weeks compared with the respective genotypes fed the isocaloric safflower oil diet (Table 1). In wild-type and PPAR- $\alpha$  null mice, dietary fish oil tended to lower plasma triacylglycerol concentrations below levels displayed by the respective genotype group fed a control diet and markedly increased plasma adiponectin concentrations (Table 1). **At 2 weeks, high-fat feeding did not impair peripheral insulin action.** Peripheral insulin action in skeletal muscles and white adipose tissue determined at the end of euglycemic-hyperinsulinemic clamp experiments was comparable between all dietary groups and genotypes (Table 2). Skeletal muscle triacylglycerol content did not differ between control, safflower oil-, and fish oil diet-fed

TABLE 2  
Euglycemic-hyperinsulinemic clamps conducted in PPAR- $\alpha$  null and wild-type mice

	Wild type			PPAR- $\alpha$ null		
	Control	Safflower oil	Fish oil	Control	Safflower oil	Fish oil
Body weight (g)	26.0 $\pm$ 0.5	25.9 $\pm$ 0.8	26.1 $\pm$ 0.8	26.9 $\pm$ 0.6	27.4 $\pm$ 0.9	26.7 $\pm$ 1.0
Basal HGP (mg $\cdot$ kg <sup>-1</sup> $\cdot$ min <sup>-1</sup> )	16.4 $\pm$ 1.5	14.7 $\pm$ 1.4	16.8 $\pm$ 2.3	16.0 $\pm$ 1.8	17.5 $\pm$ 1.5	15.9 $\pm$ 1.3
<b>Euglycemic-hyperinsulinemic clamp</b>						
$R_d$ (mg $\cdot$ kg <sup>-1</sup> $\cdot$ min <sup>-1</sup> )	32.2 $\pm$ 2.4	27.0 $\pm$ 3.1	28.8 $\pm$ 2.2	35.8 $\pm$ 3.0*	30.9 $\pm$ 2.3	24.3 $\pm$ 1.8†
Glycolysis (mg $\cdot$ kg <sup>-1</sup> $\cdot$ min <sup>-1</sup> )	21.3 $\pm$ 1.8	18.1 $\pm$ 2.4	19.7 $\pm$ 1.6	30.3 $\pm$ 3.2‡	20.6 $\pm$ 2.3†	18.2 $\pm$ 1.9†
Hepatic PEPCK mRNA expression (%18S rRNA)	100 $\pm$ 25	197 $\pm$ 14§	83 $\pm$ 19	160 $\pm$ 25	219 $\pm$ 34	164 $\pm$ 10
Plasma nonesterified fatty acids (mmol/l)	0.39 $\pm$ 0.04	0.42 $\pm$ 0.04	0.29 $\pm$ 0.05	0.57 $\pm$ 0.05	0.37 $\pm$ 0.02	0.40 $\pm$ 0.03
<b>Tissue 2-[<sup>14</sup>C]DG uptake</b>						
Gastrocnemius muscle (mg $\cdot$ kg <sup>-1</sup> $\cdot$ min <sup>-1</sup> )	138.7 $\pm$ 19.2	152.8 $\pm$ 22.3	141.3 $\pm$ 20.3	186.7 $\pm$ 20.4	172.1 $\pm$ 25.1	210.5 $\pm$ 15.0
Quadriceps muscle (mg $\cdot$ kg <sup>-1</sup> $\cdot$ min <sup>-1</sup> )	120.1 $\pm$ 12.5	139.8 $\pm$ 23.2	180.3 $\pm$ 23.0	141.0 $\pm$ 25.0	178.7 $\pm$ 14.6	180.7 $\pm$ 27.7
White adipose tissue (mg $\cdot$ kg <sup>-1</sup> $\cdot$ min <sup>-1</sup> )	4.1 $\pm$ 0.4	3.9 $\pm$ 1.1	4.1 $\pm$ 0.7	5.5 $\pm$ 0.9	3.8 $\pm$ 0.4	5.5 $\pm$ 0.9

Data are the means  $\pm$  SE of 7–10 mice per group. Male PPAR- $\alpha$  null or wild-type mice were fed either a control diet or an isocaloric high-fat diet containing 27% safflower oil without or with an 8% fish oil replacement for 2 weeks. Weight-matched mice were 6-h food deprived prior to euglycemic (~5 mmol/l)-hyperinsulinemic (2.5 mU  $\cdot$  kg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>) clamps. \* $P$  < 0.005 vs. wild-type control; † $P$  < 0.005 vs. control genotype; ‡ $P$  < 0.05 vs. wild-type control; § $P$  < 0.05 vs. control genotype.

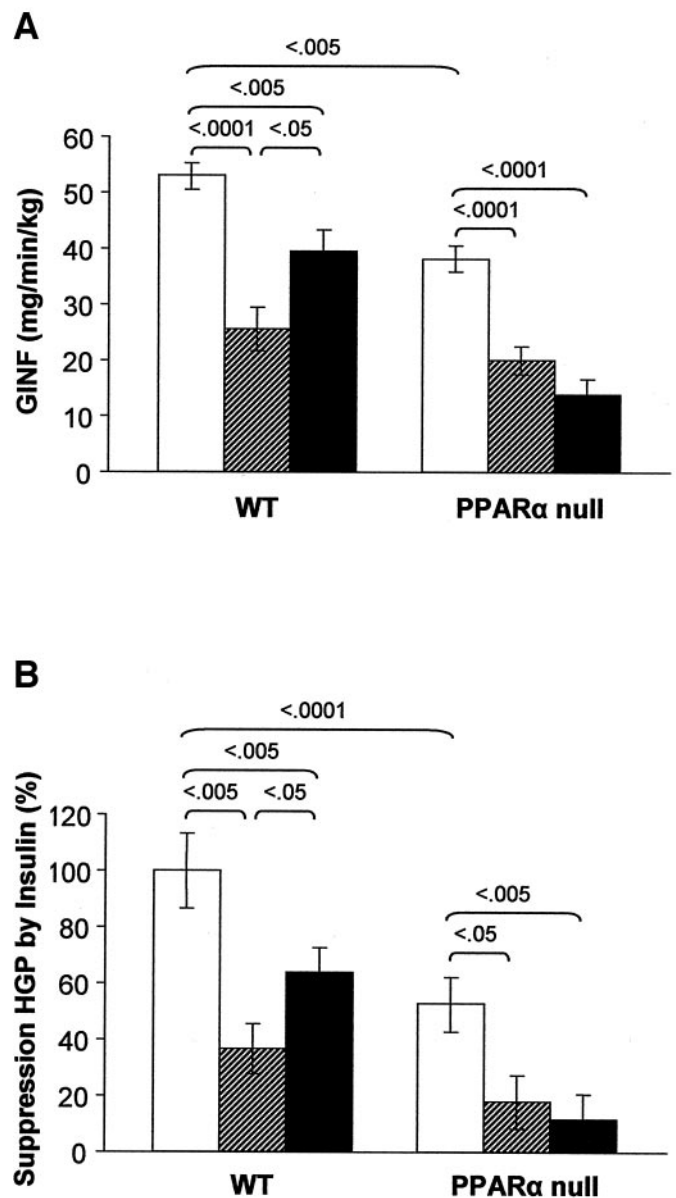


PPAR- $\alpha$  null ( $5.44 \pm 0.94$  vs.  $4.99 \pm 0.49$  vs.  $5.69 \pm 0.82$  mmol/g muscle,  $P = \text{NS}$ ,  $n = 8-9$ ) and wild-type mice ( $4.23 \pm 0.59$  vs.  $4.17 \pm 0.54$  vs.  $4.44 \pm 0.41$ ,  $P = \text{NS}$ ,  $n = 8-9$ ).

**Fish oil preserved hepatic insulin sensitivity in a PPAR- $\alpha$ -dependent fashion in body weight-matched mice.** When euglycemic-hyperinsulinemic clamp experiments were performed, 6-h fasting, control diet-fed PPAR- $\alpha$  null mice displayed  $\sim 1.4$ -fold lower glucose infusion rates than wild-type mice (Fig. 1A). Insulin's ability to suppress HGP was  $\sim 2$ -fold lower in PPAR- $\alpha$  null than wild-type mice and was paralleled by 1.6-fold increased hepatic PEPCK gene expression (Fig. 1B and Table 2). Whole-body glycolytic activity under insulin-stimulated conditions was increased by  $\sim 1.4$ -fold in control diet-fed PPAR- $\alpha$  null compared with wild-type mice (Table 2). Independent from the genotype, 2 weeks' safflower oil feeding caused severe hepatic insulin resistance paralleled by reduced glucose infusion rates and a decreased potential of insulin to suppress HGP when compared with the respective genotype fed the control diet (Fig. 1A and B and Table 2). Hepatic insulin resistance was accompanied by an increase in hepatic PEPCK expression in both genotypes (Table 2). Solely in PPAR- $\alpha$  null mice, whole-body glycolysis rates were significantly impaired compared with the respective genotype fed the control diet (Table 2). Strikingly, in wild-type mice, an 8% dietary fish oil supplement restored insulin sensitivity compared with wild-type mice treated with safflower oil and increased glucose infusion rate by 1.5-fold and insulin-mediated suppression of HGP by 2-fold (Fig. 1A and B). In sharp contrast, in PPAR- $\alpha$  null mice, 8% dietary fish oil supplementation completely failed to restore insulin sensitivity, as indicated by comparably low glucose infusion rates, and abolished insulin action on suppression of HGP compared with that observed in safflower oil-treated PPAR- $\alpha$  null mice (Fig. 1A and B).

**Fish oil counteracted hepatic triacylglycerol accumulation in a PPAR- $\alpha$ -independent fashion.** To better understand the biochemical mechanisms underlying high-fat diet-induced insulin resistance, we first measured hepatic triacylglycerol content. Feeding mice the 27% safflower oil diet for 2 weeks raised hepatic triacylglycerol concentrations approximately fourfold in both genotypes (Table 3). In both wild-type and PPAR- $\alpha$  null mice, dietary fish oil lowered intrahepatic triacylglycerol concentrations approximately twofold when compared with the respective genotype fed the isocaloric 27% safflower oil diet (Table 3).

**Reductions in hepatic triacylglycerol concentrations did not rescue hepatic insulin sensitivity in PPAR- $\alpha$  null mice.** To rule out hepatic triacylglycerol accumulation as a mediator of hepatic insulin resistance, hepatic triacylglycerol formation was limited by feeding PPAR- $\alpha$  null mice the 27% safflower oil diet for only 1 week. The dietary regimen lead to hepatic triacylglycerol concentrations markedly below those displayed by 2 weeks' safflower oil diet-fed PPAR- $\alpha$  null mice ( $28.4 \pm 4.0$  vs.  $85.5 \pm 5.9$  mmol/g liver,  $n = 6$  vs. 9). However, decreased hepatic triacylglycerol deposition did not rescue insulin action in PPAR- $\alpha$  null mice compared with 2 weeks' safflower oil diet-fed PPAR- $\alpha$  null mice (glucose infusion rate:  $18.8 \pm 4.1$  vs.  $20.0 \pm 2.5$  mg  $\cdot$  kg $^{-1}$   $\cdot$  min $^{-1}$ ; insulin-mediated suppression HGP:  $4.6 \pm 5.0$  vs.  $7.2 \pm 1.9$  mg  $\cdot$  kg $^{-1}$   $\cdot$  min $^{-1}$ ;  $n = 6$  vs. 8).



**FIG. 1.** Glucose infusion rate (GINF) (A) and suppression of HGP (B) under insulin-stimulated conditions in PPAR- $\alpha$  null and wild-type (WT) mice. Euglycemic-hyperinsulinemic clamp experiments were conducted in male PPAR- $\alpha$  null and wild-type mice fed for 2 weeks with either a control diet or isocaloric high-fat diets containing safflower oil without or with an 8% fish oil replacement. Data are the means  $\pm$  SE of 7–10 mice per group.  $\square$ , control diet;  $\square$  with diagonal lines, isocaloric high-fat diets containing safflower oil without fish oil;  $\blacksquare$ , isocaloric high-fat diets containing safflower oil with fish oil.

**Fish oil decreased total hepatic acyl-CoA concentrations in a PPAR- $\alpha$ -dependent fashion.** As shown in Fig. 2A, hepatic insulin resistance was not associated with increases in total hepatic acyl-CoA concentrations. In fish oil-fed PPAR- $\alpha$  null mice, hepatic insulin resistance was accompanied by a marked decline in both saturated and unsaturated hepatic acyl-CoA concentrations (Fig. 2A). Individual hepatic acyl-CoA species reflected the fatty acid composition of the respective dietary oils. Fish oil consumption increased the abundance of n-3 type acyl-CoA species almost threefold in PPAR- $\alpha$  null mice and twofold in wild-type mice (Fig. 2C and Table 3), and safflower oil consumption increased the acyl-CoA derivative of C18:2n-6 almost threefold in PPAR- $\alpha$  null mice and more than

TABLE 3  
Liver weight and hepatic lipid metabolite profile of PPAR- $\alpha$  null and wild-type mice

	Wild type			PPAR- $\alpha$ null		
	Control	Safflower oil	Fish oil	Control	Safflower oil	Fish oil
Liver weight (% body wt)	3.26 $\pm$ 0.09	3.47 $\pm$ 0.08	3.47 $\pm$ 0.10	3.40 $\pm$ 0.14	4.47 $\pm$ 0.14*	3.93 $\pm$ 0.09†‡
Liver triacylglycerol (mmol/g)	6.24 $\pm$ 1.33	23.22 $\pm$ 5.76†	13.44 $\pm$ 2.37	23.12 $\pm$ 4.24§	85.47 $\pm$ 5.91*	51.39 $\pm$ 6.11‡
Liver acyl-CoA species (all measured species)						
Total very-long-chain acyl-CoA (nmol/g)	6.23 $\pm$ 0.87	8.70 $\pm$ 1.86	15.26 $\pm$ 2.30	6.12 $\pm$ 1.04	15.48 $\pm$ 4.58	10.88 $\pm$ 2.86
C16:0 (nmol/g)	12.06 $\pm$ 1.54	8.89 $\pm$ 1.15	9.90 $\pm$ 1.35	14.30 $\pm$ 3.05	7.75 $\pm$ 1.63	4.88 $\pm$ 0.55
C16:1 (nmol/g)	10.17 $\pm$ 1.45	5.27 $\pm$ 1.10†	5.51 $\pm$ 0.94†	11.64 $\pm$ 2.62	4.31 $\pm$ 1.15†	2.22 $\pm$ 0.42
C18:0 (nmol/g)	11.59 $\pm$ 1.55	12.45 $\pm$ 1.72	11.45 $\pm$ 1.12	14.55 $\pm$ 1.72	13.21 $\pm$ 2.42	6.96 $\pm$ 0.91‡
C18:1 (nmol/g)	22.83 $\pm$ 4.16	20.76 $\pm$ 2.37	17.92 $\pm$ 1.95	30.27 $\pm$ 3.09	26.86 $\pm$ 4.33	12.66 $\pm$ 1.62‡
C18:2n-6 (nmol/g)	19.83 $\pm$ 1.68	46.18 $\pm$ 7.03	38.30 $\pm$ 2.13*	21.78 $\pm$ 1.74	61.74 $\pm$ 17.98	26.17 $\pm$ 5.21
C18:3n-3 (nmol/g)	6.02 $\pm$ 0.57	6.21 $\pm$ 0.87	8.33 $\pm$ 1.24	7.83 $\pm$ 0.71	6.75 $\pm$ 1.09	4.03 $\pm$ 0.64‡
C20:0 (nmol/g)	1.16 $\pm$ 0.25	1.24 $\pm$ 0.11	1.55 $\pm$ 0.32	1.31 $\pm$ 0.37	1.50 $\pm$ 0.36	0.75 $\pm$ 0.13‡
C20:5n-3 (nmol/g)	2.91 $\pm$ 0.43	5.07 $\pm$ 1.22	10.32 $\pm$ 1.43‡	3.38 $\pm$ 0.59	12.25 $\pm$ 4.06	6.99 $\pm$ 1.65
C22:0 (nmol/g)	0.25 $\pm$ 0.03	0.44 $\pm$ 0.06†	0.53 $\pm$ 0.16	0.36 $\pm$ 0.17	0.33 $\pm$ 0.10	0.28 $\pm$ 0.06
C22:6n-3 (nmol/g)	2.78 $\pm$ 0.28	3.07 $\pm$ 0.48	5.11 $\pm$ 0.38‡	1.82 $\pm$ 0.10§	3.89 $\pm$ 1.25	5.47 $\pm$ 1.32†
C24:0 (nmol/g)	0.07 $\pm$ 0.01	0.16 $\pm$ 0.05	0.16 $\pm$ 0.01	0.07 $\pm$ 0.02	0.12 $\pm$ 0.02	0.14 $\pm$ 0.03
C26:0 (nmol/g)	0.01 $\pm$ 0.01	0.08 $\pm$ 0.03†	0.16 $\pm$ 0.05†	0.05 $\pm$ 0.02	0.09 $\pm$ 0.02	0.02 $\pm$ 0.01‡
Liver diacylglycerol species (selected species)						
C16:0-C16:0 ( $\mu$ mol/g)	0.48 $\pm$ 0.24	0.97 $\pm$ 0.33	0.65 $\pm$ 0.30	0.65 $\pm$ 0.40	1.02 $\pm$ 0.51	2.34 $\pm$ 0.92
C18:0-C18:0 ( $\mu$ mol/g)	0.32 $\pm$ 0.07	3.39 $\pm$ 1.37	1.79 $\pm$ 0.95	1.45 $\pm$ 1.11	2.55 $\pm$ 1.38	5.92 $\pm$ 2.58
C18:1-C18:1 ( $\mu$ mol/g)	1.06 $\pm$ 0.31	0.37 $\pm$ 0.17	0.29 $\pm$ 0.07†	1.08 $\pm$ 0.26	0.83 $\pm$ 0.23	2.26 $\pm$ 0.46‡
C18:1-C18:2 ( $\mu$ mol/g)	0.64 $\pm$ 0.14	0.77 $\pm$ 0.31	0.51 $\pm$ 0.11	1.09 $\pm$ 0.18	2.87 $\pm$ 0.72†	4.88 $\pm$ 1.07
C18:2-C18:2 ( $\mu$ mol/g)	0.47 $\pm$ 0.09	1.87 $\pm$ 0.86	1.03 $\pm$ 0.19†	1.05 $\pm$ 0.18§	9.25 $\pm$ 2.10†	9.80 $\pm$ 2.94
C18:2-C20:4 ( $\mu$ mol/g)	0.03 $\pm$ 0.01	0.07 $\pm$ 0.02	0.03 $\pm$ 0.01	0.06 $\pm$ 0.01	0.29 $\pm$ 0.05	0.27 $\pm$ 0.05
C18:2-C22:6 ( $\mu$ mol/g)	0.06 $\pm$ 0.02	0.07 $\pm$ 0.04	0.16 $\pm$ 0.03	0.15 $\pm$ 0.03§	0.24 $\pm$ 0.04	2.63 $\pm$ 1.07†‡
C18:2-C20:5 ( $\mu$ mol/g)	0.07 $\pm$ 0.02	0.11 $\pm$ 0.03	0.09 $\pm$ 0.02	0.11 $\pm$ 0.03	0.18 $\pm$ 0.02	0.83 $\pm$ 0.18†‡
C20:5-C20:5 ( $\mu$ mol/g)	0.13 $\pm$ 0.03	0.13 $\pm$ 0.04	0.22 $\pm$ 0.05	0.17 $\pm$ 0.03	0.27 $\pm$ 0.03†	0.66 $\pm$ 0.19†
C20:5-C20:6 ( $\mu$ mol/g)	0.05 $\pm$ 0.02	0.05 $\pm$ 0.02	0.05 $\pm$ 0.01	0.09 $\pm$ 0.02	0.17 $\pm$ 0.02†	0.32 $\pm$ 0.09†

Data are the means  $\pm$  SE of four to seven mice per group. Male PPAR- $\alpha$  null and wild-type mice were fed either a control diet or isocaloric high-fat diets containing 27% safflower oil without or with an 8% fish oil replacement for 2 weeks. The 6-h food-deprived mice were anesthetized, livers freeze-clamped in situ, and lipid metabolites extracted for LC/MS/MS analysis of acyl-CoA, diacylglycerol, and triacylglycerol analysis. \* $P$  < 0.0001, † $P$  < 0.05 vs. control genotype; ‡ $P$  < 0.05 vs. safflower genotype; § $P$  < 0.05 vs. wild-type control; || $P$  < 0.005 vs. control genotype.

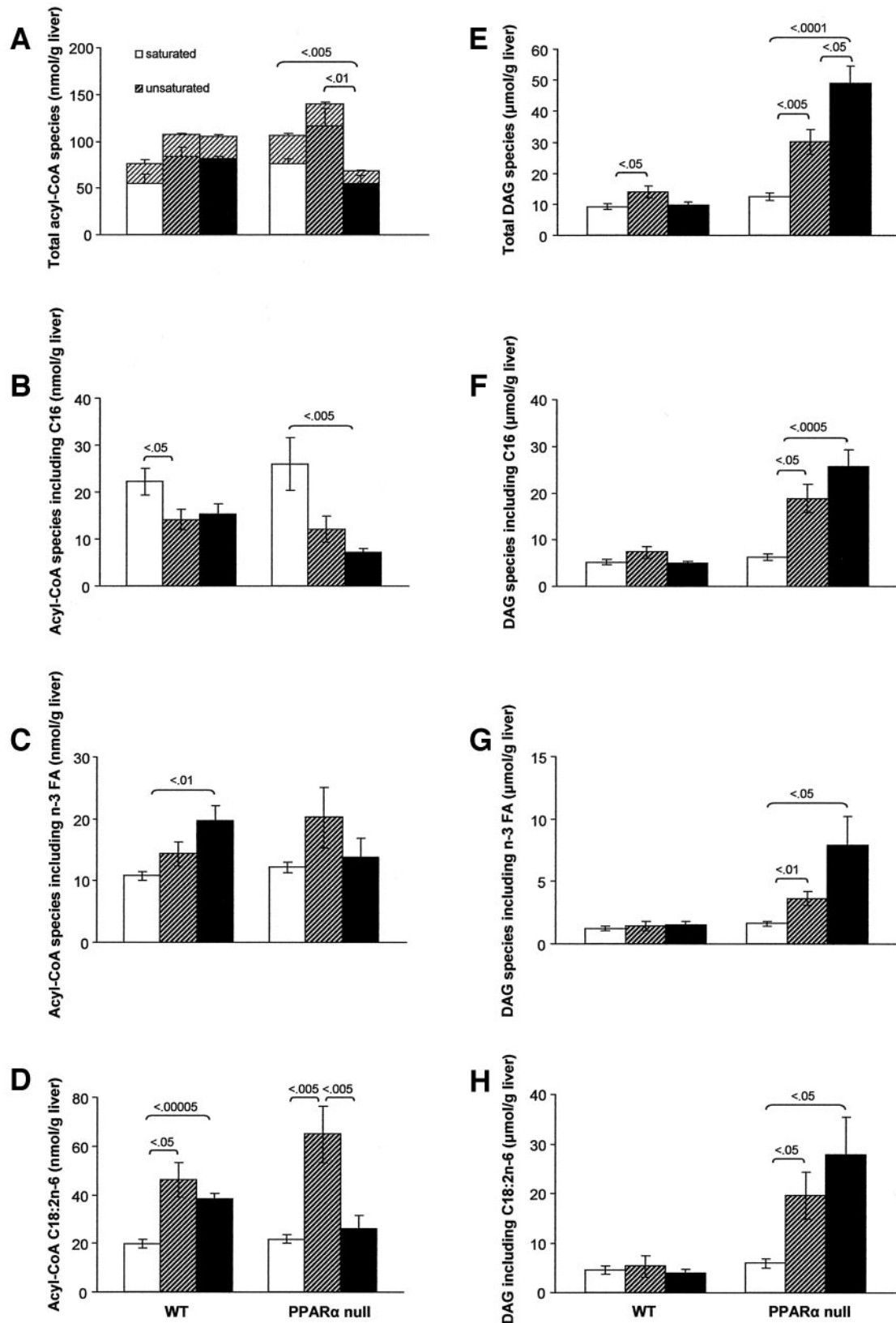
twofold in wild-type mice (Fig. 2D and Table 3) compared with the respective genotypes fed control diet. In the absence of functional PPAR- $\alpha$ , dietary fish oil did not increase total hepatic very-long-chain acyl-CoA concentrations (C20:5n-3, C22:6n-3, C22:0-, C24:0-, and C26:0-CoA), but in wild-type mice, it led to an approximately twofold increase compared with the respective genotypes fed safflower oil or control diet (Table 3).

**Fish oil elevated total hepatic diacylglycerol species in a PPAR- $\alpha$ -dependent fashion.** As a consequence of safflower oil feeding, hepatic diacylglycerol concentrations increased 1.5-fold in wild-type mice and close to 2.5-fold in PPAR- $\alpha$  null mice compared with the respective genotype fed control diet (Fig. 2E and Table 3). Dietary safflower oil raised the diacylglycerol species C18:2n-6/C18:2n-6 (9-fold) and C18:2n-6/C20:4n-6 (5-fold) in PPAR- $\alpha$  null mice and C18:0/C18:0 (11-fold) and C18:2n-6/C18:2n-6 (4-fold) in wild-type mice to the most pronounced extent (Table 3). In wild-type mice, 8% dietary fish oil abolished increases in total hepatic diacylglycerol concentrations compared with mice of the same genotype fed the isocaloric safflower oil diet (Fig. 2E). In sharp contrast, in

PPAR- $\alpha$  null mice, dietary fish oil increased total hepatic diacylglycerol concentrations almost 4-fold, with individual species elevated from a minimum 1.2-fold to a maximum 18-fold (C18:2n-6/C22:6n-3 diacylglycerol) above concentrations observed in control diet-fed PPAR- $\alpha$  null mice (Fig. 2E and Table 3). When fish oil was fed to PPAR- $\alpha$  null mice, they displayed the most pronounced increases in diacylglycerol species containing C18:2n-6, C20:5n-3, and C22:6n-3 compared with the respective genotype fed the control diet (Fig. 2G and H and Table 3).

## DISCUSSION

The major observation in the current study is that fish oil prevented fat-induced hepatic insulin resistance in vivo in a PPAR- $\alpha$ -dependent manner. Consistent with previous reports, this study found that fish oil increased plasma adiponectin concentrations (24,25), which has been shown to reverse insulin resistance in rodents in part by increasing hepatic insulin sensitivity (26–28). However, this study demonstrates that fish oil-induced increases in plasma adiponectin concentrations were not sufficient to pro-



**FIG. 2.** Acyl-CoA (A–D) and diacylglycerol (DAG) (E–H) species in livers of PPAR- $\alpha$  null and wild-type (WT) mice. Male PPAR- $\alpha$  null or wild-type mice were fed for 2 weeks with either a control diet or isocaloric high-fat diets containing 27% safflower oil without or with an 8% fish oil replacement, livers freeze-clamped in situ, and lipid metabolites extracted for LC/MS/MS analysis. Total acyl-CoA (A) and diacylglycerol (E) content are displayed as well as selected acyl-CoA (B–D) and diacylglycerol species (F–H). Results are the means  $\pm$  SE of six mice per group. B–H:  $\square$ , control diet;  $\square$  with diagonal lines, isocaloric high-fat diets containing safflower oil without fish oil;  $\blacksquare$ , isocaloric high-fat diets containing safflower oil with fish oil.



tect PPAR- $\alpha$  null mice from fat-induced hepatic insulin resistance.

The accumulation of various lipid metabolites in liver and skeletal muscles has been linked with the pathogenesis of insulin resistance (4–7,29–33). Fish oil-mediated activation of PPAR- $\alpha$  has been shown to induce peroxisome proliferation and the concerted transcriptional upregulation of genes involved in mitochondrial and peroxisomal fatty acid oxidation (5,34). An enhanced hepatic fatty acid oxidation capacity likely partitions lipid metabolites preferentially toward degradative pathways, decreasing their intracellular abundance (4,5,12,14,34). Fish oil also regulates the prolipogenic transcription factor sterol regulatory element binding protein (SREBP)-1 by accelerating the rate of SREBP-1 mRNA decay and decreasing SREBP-1 protein maturation (35,36). The data in the current study provide further evidence that, independent from PPAR- $\alpha$ , fish oil consumption likely restricts hepatic triacylglycerol deposition by mediating a decrease in intracellular SREBP-1 abundance and subsequently the expression of SREBP-modulated prolipogenic genes. Alternatively, decreased intracellular triacylglycerol abundance could have resulted from fish oil-mediated alterations in the membrane saturated-to-monounsaturated fatty acid ratio and consequently membrane fluidity. Such “leaky” membranes might lead to dissipation of energy derived from increased fat oxidation for maintenance of cellular integrity (37,38).

Increases in intracellular long-chain acyl-CoA esters have been linked to the development of insulin resistance in the past (33). In the current study, fish oil-fed PPAR- $\alpha$  null mice displayed the lowest total hepatic long-chain acyl-CoA concentrations among all groups, arguing against a direct causative role of long-chain acyl-CoA concentrations in hepatic insulin resistance. These data are consistent with recent findings in high-fat diet-fed mtGPAT1 knockout mice that displayed markedly elevated hepatic acyl-CoA concentrations, despite improved hepatic insulin sensitivity, when compared with wild-type mice (6).

In contrast to triacylglycerol and acyl-CoA concentrations, hepatic diacylglycerol concentrations were the most consistent predictors of hepatic insulin sensitivity in the current study. Feeding mice the safflower oil diet increased hepatic diacylglycerol abundance, and, in the absence of functional PPAR- $\alpha$ , fish oil feeding caused a large increase in hepatic diacylglycerol content. Diacylglycerol has been shown to activate protein kinase C- $\epsilon$  in liver, which in turn inhibits insulin receptor kinase activity (6,7,33,39). These data further support a key role of diacylglycerol in mediating hepatic insulin resistance.

PPAR- $\alpha$  activation is critical in the adaptive response to fasting, and, in the course of food deprivation, PPAR- $\alpha$  null mice display severely impaired fatty acid oxidation, ketogenic capacity, elevated plasma free fatty acid concentrations, hypoglycemia, and hypothermia (40,41). In the current study, PPAR- $\alpha$  null mice fed control chow displayed hypoglycemia after only 6 h of fasting. Consistent with previous studies, hypoglycemia in these mice appears to be driven by increased peripheral glucose utilization, compensating for impaired fatty acid oxidation capacity rather than impaired HGP (42,43). An increased glycolytic flux in pancreatic  $\beta$ -cells of PPAR- $\alpha$  null mice resulting from impaired fatty acid oxidation capacity could also explain the observed increases in plasma insulin concentrations. Tordjman et al. (44) reported that treatment of INS-1 cells with the PPAR- $\alpha$  agonist clofibric acid de-

creased both basal and glucose-stimulated insulin secretion, whereas etomoxir, a carnitine palmitoyl transferase 1 and fatty acid  $\beta$ -oxidation inhibitor, prevented clofibric acid-induced suppression of glucose-stimulated insulin secretion.

In summary, fish oil prevents fat-induced hepatic insulin resistance through a PPAR- $\alpha$ -dependent pathway. Hepatic diacylglycerol content serves as the most consistent predictor of hepatic insulin sensitivity, whereas both hepatic acyl-CoA and triacylglycerol concentrations were uncoupled from hepatic insulin resistance, providing further evidence that these lipid metabolites do not play a direct causative role in mediating this process.

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