

Fish oil and indomethacin in combination potently reduce dyslipidemia and hepatic steatosis in LDLR^{-/-} mice[§]

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Abstract Fish oil (FO) is a potent anti-inflammatory and lipid-lowering agent. Because inflammation can modulate lipid metabolism and vice versa, we hypothesized that combining FO with cyclooxygenase inhibitors (COXIBs), well-known anti-inflammatory drugs, can enhance the anti-inflammatory and lipid-lowering effect of FO. LDLR^{-/-} mice were fed a high-fat diet supplemented with 6% olive oil or FO for 12 wk in the presence or absence of indomethacin (Indo, 6 mg/l drinking water). FO reduced plasma total cholesterol by 30% but, in combination with Indo, exerted a greater decrease (44%). The reduction of liver cholesterol ester (CE) and triglycerides (TG) by FO (63% and 41%, respectively) was enhanced by Indo (80% in CE and 64% in TG). FO + Indo greatly increased the expression of genes modulating lipid metabolism and reduced the expression of inflammatory genes compared with control. The mRNA and/or protein expression of pregnane X receptor (PXR) and cytochrome P450 isoforms that alter inflammation and/or lipid metabolism are increased to a greater extent in mice that received FO + Indo. Moreover, the nuclear level of PXR is significantly increased in FO + Indo group. Combining FO with COXIBs may exert their beneficial effects on inflammation and lipid metabolism via PXR and cytochrome P450.—Murali, G., G. L. Milne, C. D. Webb, A. B. Stewart, R. P. McMillan, B. C. Lyle, M. W. Hulver, and V. Saraswathi. Fish oil and indomethacin in combination potently reduce dyslipidemia and hepatic steatosis in LDLR^{-/-} mice. *J. Lipid Res.* 2012. 53: 2186–2197.

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Dyslipidemia, which is characterized by elevated cholesterol or triglycerides, is a major risk factor for coronary heart disease (as reviewed in Ref. 1). One of the most studied forms of dyslipidemia is familial hypercholesterolemia (FH) caused by genetic factors. Although homozygous FH is a rare condition, heterozygous FH occurs in approximately 1 in 500 people and can cause premature atherosclerotic disease (as reviewed in Ref. 2). Combination therapy is commonly used in clinical practice to improve hypercholesterolemia. The most commonly used combination therapy consists of statins with fibrates or niacin. However, this combination has limited additive effect on LDL cholesterol levels. Moreover, the poor tolerance of niacin and the statin-induced side effects (e.g., muscle toxicity) limit the use of this combination (as reviewed in Refs. 3 and 4). In addition to dyslipidemia, inflammation by itself can lead to increased risk of coronary heart disease. Therefore, a therapeutic regimen that has the potential to reduce dyslipidemia and inflammation would be more beneficial in reducing cardiovascular events than treatments aimed at improving dyslipidemia alone.

Fish oil (FO) containing n-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid

Abbreviations: AA, arachidonic acid; ACOT-3, AcylCoA thioesterase-3; ASM, acid-soluble metabolite; AT, adipose tissue; BAT, brown adipose tissue; CE, cholesterol ester; COXIB, cyclooxygenase inhibitors; CYP 450, cytochrome P450; FASN, fatty acid synthase; FH, familial hypercholesterolemia; FO, fish oil; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; TC, total cholesterol; TG, triglyceride; TX, thromboxane.

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is a potent anti-inflammatory (as reviewed in Ref. 5) and triglyceride (TG)-lowering agent (6). In addition to its TG-lowering effect, FO has been reported to reduce LDL cholesterol levels in patients with FH and normal subjects (7). Case reports have shown that FO may be beneficial to children with FH (8). Moreover, we (9) and others (10) have shown that FO reduces plasma cholesterol levels in LDLR^{-/-} mice, a model for FH. Although FO is a safe alternative to other hypolipidemic agents, it exerts a modest cholesterol-lowering effect, and therefore very high doses are needed. Thus, it is important to develop a strategy to improve the therapeutic efficacy of FO.

The liver plays a central role in regulating plasma lipid levels, and it is becoming clear that inflammatory events in the liver may modulate hepatic lipid metabolism. For example, it has been shown that the increased inflammatory response in liver resident macrophages or Kupffer cells promotes the development of hepatic steatosis and that elimination of these activated resident Kupffer cells is associated with reduced hepatic steatosis (11, 12). Furthermore, several anti-inflammatory agents have been shown to promote hepatic lipid metabolism. For example, dietary components with known anti-inflammatory properties (e.g., curcumin, baicalin, and capsaicin) have been shown to ameliorate metabolic disorders and hepatic steatosis (13–15). Regarding dietary FO, another anti-inflammatory agent, we and others have reported that FO or the n-3 fatty acids can ameliorate hepatic steatosis (9, 16) and improve dyslipidemia. Taken together, these reports suggest that several anti-inflammatory agents have the propensity to promote lipid catabolism in liver.

FO and Indo mediate their anti-inflammatory effects by modulating arachidonic acid (AA) metabolism. For example, Indo inhibits COX-mediated production of 2-series eicosanoids from AA, which are mostly proinflammatory. Alternatively, the n-3 fatty acids replace AA from the membrane phospholipids, thereby reducing the availability of AA to COX and thus reducing the production of 2-series eicosanoids from AA. The n-3 fatty acids by themselves can be metabolized by COX, giving rise to 3-series eicosanoids that are anti-inflammatory or less proinflammatory compared with AA-derived 2-series eicosanoids (17). Overall, the n-3 fatty acids and cyclooxygenase inhibitors (COXIBs) act through modulating AA metabolism. Because of their overlapping effect on AA metabolism, it was postulated that a combination of n-3 fatty acids and (COXIBs) may exert an enhanced anti-inflammatory effect. In fact, a synergistic anti-inflammatory effect of this combination has been reported in monocytes (18). Moreover, Serhan et al. (19) have shown that aspirin, a COX inhibitor, triggers the formation of potent anti-inflammatory agents called resolvins from n-3 fatty acids. However, the efficacy of this combined therapy in modulating hepatic inflammatory response and/or lipid accumulation is unknown. Because inflammation can modulate lipid metabolism, we hypothesized that combining fish oil with COXIBs may exert enhanced anti-inflammatory and lipid-lowering effects.

LDLR^{-/-} mice, a model for FH, are widely used to study dyslipidemia and atherosclerosis. Using this model, we

studied the effects of dietary FO on hepatic steatosis and dyslipidemia in the presence or absence of Indo, an isoform-nonspecific COXIB. We demonstrate that FO in the presence of Indo exerts synergistic anti-inflammatory and lipid-lowering effects in the liver, which is associated with enhanced hypolipidemic effects in plasma. The findings will have implications in developing a therapeutic strategy to improve the efficacy of dietary FO in alleviating dyslipidemia and hepatic steatosis in hyperlipidemic patients who are at an increased risk of premature mortality due to cardiovascular disease.

MATERIALS AND METHODS

Mice

LDLR^{-/-} mice originally obtained from Jackson Laboratories were used in the present study. Female LDLR^{-/-} mice (2–3 mo old) were fed a high-fat diet (39% KJ from fat) supplemented with 6% (by weight) olive oil or FO and 0.5% cholesterol for 12 wk in the presence or absence of Indo (6 mg/l) (20) in drinking water. A stock of Indo was made in DMSO, which was then mixed in drinking water. The water containing Indo was changed every day. The olive oil-fed mice and FO alone-fed mice received DMSO as a vehicle control in their drinking water. We have published the composition of the diet in detail previously (9). Cumulative food intake was measured in two cages of mice per group. At the end of 12 wk, the mice were fasted for 5 h and euthanized by isoflurane overdose followed by cervical dislocation. All animal care procedures were carried out with approval from the Institutional Animal Care and Use Committee of Vanderbilt University, Virginia Tech, and VA Nebraska Western Iowa Health Care System, Omaha, Nebraska.

Plasma measurements

Plasma total cholesterol (TC) and triglycerides (TGs) were analyzed using kits from Raichem. Plasma FFAs were measured using kits from Wako Chemicals. Plasma levels of cholesterol ester (CE), free cholesterol, and phospholipids were analyzed by GC at the Lipid Core Laboratory. Blood glucose was measured using a Lifescan glucometer from Johnson and Johnson. Plasma insulin measurements were performed using an insulin assay kit (Linco Research). The homeostasis model assessment of insulin resistance (HOMA-IR) was used as a measure of insulin resistance and was calculated using the following equation: HOMA-IR = fasting serum insulin (μ U/ml) \times fasting serum glucose (mg/dl)/405. Plasma lipoprotein profiles were analyzed by fast protein liquid chromatography using a superose 6 column (Amersham Pharmacia). Pooled plasma samples (100 μ l) were loaded onto the column, and 40 fractions (0.5 ml each) were collected for cholesterol measurement. Fractions 15–20 contained VLDL, fractions 21–26 contained LDL, and fractions 27–34 contained HDL.

Lipid analysis

The levels of cholesterol ester (CE) and TGs in liver samples were determined by GC at the Lipid Core Laboratory of Vanderbilt University.

Microarray analysis

The microarray analysis of liver RNA samples were carried out using pooled RNA samples (six samples per group) at the Functional Genomics Shared Resources at Vanderbilt University. Further details for the experimental procedures are provided in the supplementary material.

Real-time PCR

Total RNA was extracted from liver, perigonadal adipose tissue, and brown adipose tissue (BAT) samples using TRIzol reagent (Invitrogen), and cDNA synthesis was carried out using iScript cDNA synthesis kit from Bio-rad. Real-time PCR analysis was performed for genes involved in lipid metabolism and inflammatory response using applied on-demand primer-probes from Applied Biosystems. We have used the $\Delta\Delta$ CT method to quantify the mRNA expression levels. Except for low-expression genes (inflammatory genes), we set the number of cycles as 36.

Western blot analysis

Liver samples were homogenized in lysis buffer containing 20 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 1 mmol/l ethylenediaminetetraacetic acid, 1 mmol/l ethylene glycol tetraacetic acid, 0.5% NP-40, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l sodium orthovanadate, and protease inhibitor cocktail (Roche). The gels were immunoblotted for PXR, CYP 3A, and CYP 4A. To confirm equal loading, blots were also probed with GAPDH antibody. Because the isoform-specific antibodies for CYP 3A4 and 4A10 are not available, we used general antibodies for CYP 3A and CYP 4A in our study. We purchased the antibodies for pregnane X receptor (PXR), CYP 3A, and CYP 4A from Santacruz Biotech. GAPDH antibody was purchased from Cell Signaling.

Preparation of nuclear extracts

The nuclear extracts were prepared from liver samples as reported previously (21). We performed Western blot analysis using the nuclear extracts to determine the nuclear translocation of peroxisome proliferator-activated receptor (PPAR) α and PXR using anti-PPAR α and anti-PXR antibodies, respectively (Santa Cruz Biotechnology). We used TATA binding protein as a loading control, and anti-TATA binding protein antibody was purchased from ABCAM.

Measures of fatty acid oxidation

Fatty acid oxidation was measured in whole liver homogenates by measuring and summing $^{14}\text{CO}_2$ production and ^{14}C -labeled acid-soluble metabolites from the oxidation of [^{14}C]palmitic acid as previously described (22–24).

TG secretion rate

The TG secretion rate was measured as described elsewhere (25). At the end of the experimental period, the mice were fasted overnight, and baseline blood samples were collected from anesthetized mice through tail vein bleeding. The mice were then injected intravenously with tyloxapol (Triton), a lipoprotein lipase inhibitor (Sigma) at a concentration of 500 mg/kg body weight. The blood samples were collected from tail veins every 1 h to 3 h, and plasma was isolated for TG analysis. The slope of the line from 0 h to 3 h was used to calculate TG production rate.

Eicosanoid measurements

Eicosanoids, such as prostaglandin (PG) E_2 , PGE $_3$, thromboxane (TX) B_2 , and TXB $_3$, in liver were analyzed by GC-MS at the Eicosanoid Core Laboratory of Vanderbilt University. Details of this experiment are provided in the supplementary material.

Measurement of COX-1 activity in whole blood

COX-1 activity in whole blood was measured as previously described (26). Briefly, 200 μl of blood samples collected in heparin (19 units/ml) at the time of death were stimulated with ionomycin

(50 μM) for 30 min at 37°C and centrifuged at 3,000 rpm for 5 min at 4°C. The plasma samples were collected and frozen at –80°C immediately. Analysis of TXB $_2$ and TXB $_3$ was carried out using GC/MS at the Eicosanoid Core Laboratory at Vanderbilt University as described in the supplementary material.

Statistical analysis

Values are presented as the mean \pm SEM. Data were analyzed with Prism Graphpad using one way ANOVA followed by Tukey's post hoc test to compare the responses among different groups. A statistical probability of $P < 0.05$ was considered significant.

RESULTS

Effect of dietary FO in the presence or absence of Indo on metabolic parameters

The body weight and liver weight of the mice did not change in any of the groups compared with control mice. The cumulative food intake was not altered much among different groups. The fat mass as measured by NMR trended toward an increase in FO and FO + Indo groups. However, the adiposity (% body weight) showed a significant increase in FO-fed mice compared with olive oil fed-control and Indo-treated mice. The adiposity was not altered in the Indo-treated mice, but the mice that received a combination of FO and Indo exhibited a trend toward an increase in adiposity. The perigonadal adipose tissue (AT) did not change in FO- or Indo-treated mice, but it increased significantly in mice that received both FO and Indo. The blood glucose, plasma insulin, and HOMA-IR were not changed in any of the groups (Table 1). Plasma TC was significantly decreased in mice that received FO ($P < 0.001$) or Indo ($P < 0.01$) compared with olive oil-fed control mice. A more potent reduction in plasma TC was seen in mice that received both FO and Indo. In fact, TC level was significantly reduced in FO + Indo-treated mice compared with mice that received only the FO diet ($P < 0.05$). The CE level showed only a trend toward a decrease in FO-fed mice. However, a significant reduction in CE was noted in mice that received FO + Indo compared with olive oil-fed control mice. Thus, mice receiving both FO and Indo showed more than 20% reduction in plasma TC and CE compared with mice that received FO only. Plasma TG levels were decreased significantly in FO- ($P < 0.001$), Indo- ($P < 0.01$), and FO + Indo-treated ($P < 0.001$) mice. Thus, although the cholesterol-lowering effect of FO was enhanced by combining with Indo, its TG-lowering effect was not altered. The plasma FFA levels were reduced significantly by FO in the absence ($P < 0.001$) or presence ($P < 0.01$) of Indo (Table 1). These data indicate that although FO by itself exerts a potent cholesterol-lowering effect in plasma, this effect is potentiated by Indo in LDLR $^{-/-}$ mice. In addition, analysis of the lipoprotein profile revealed that FO reduced VLDL and LDL cholesterol levels in LDLR $^{-/-}$ mice. Indo by itself showed a moderate decrease in VLDL and LDL cholesterol levels. In line with the plasma lipid profile, the levels of VLDL and LDL cholesterol were decreased to a greater extent in mice that received both FO and Indo (Fig. 1).

TABLE 1. Effect of FO and/or Indo on metabolic parameters in LDLR^{-/-} mice

Measurements	Olive Oil	FO	Olive Oil + Indo	FO + Indo
Body weight (g)	23.6 ± 0.8	24.7 ± 0.8	23.7 ± 0.5	24.2 ± 1.0
Liver weight (g)	1.43 ± 0.10	1.38 ± 0.06	1.58 ± 0.05	1.41 ± 0.07
Cumulative food intake (g/mouse/wk)	52.5 ± 2.9	60.0 ± 4.1	53.2 ± 3.3	50.2 ± 1.3
Fat mass (g)	3.09 ± 0.31	4.41 ± 0.38	2.89 ± 0.23	4.59 ± 0.78
Adiposity (% body weight)	12.6 ± 1.0	19.0 ± 1.5 ^d	12.8 ± 1.1	17.8 ± 2.3
Perigonadal fat mass (g)	0.34 ± 0.03	0.52 ± 0.06	0.37 ± 0.03	0.59 ± 0.10 ^a
Glucose (mg/dl)	231 ± 23	195 ± 17	225 ± 13	246 ± 23
Insulin (µg/l)	0.47 ± 0.06	0.52 ± 0.05	0.54 ± 0.05	0.54 ± 0.06
HOMA-IR	6.12 ± 0.92	6.74 ± 0.78	8.02 ± 1.19	7.96 ± 1.32
TC (mg/dl)	572 ± 21	393 ± 24 ^b	469 ± 19 ^a	311 ± 19 ^{bc}
CE (mg/dl)	638 ± 43	489 ± 38	651 ± 42	378 ± 35 ^a
Unesterified cholesterol (mg/dl)	210 ± 23	150 ± 9.7	194 ± 37	105 ± 11.7
TG (mg/dl)	209 ± 18	84 ± 8 ^b	151 ± 12 ^b	87 ± 8 ^b
Phospholipids (mg/dl)	386 ± 23	363 ± 56	341 ± 22	264 ± 23
FFAs (mEq/l)	0.87 ± 0.06	0.47 ± 0.02 ^b	0.94 ± 0.07	0.62 ± 0.03 ^a

^a*P* < 0.01 and ^b*P* < 0.001 versus olive oil; ^c*P* < 0.05 versus FO; ^d*P* < 0.05 versus olive oil and olive oil + Indo. Values are presented as mean ± SEM from 11–15 mice in each group for all the analyses except food intake, CE, UC, and phospholipids. Food intake was measured in two cages in each group and CE, UC, and phospholipids were analyzed using GC method and values are mean ± SEM of four to six samples in each group.

Effect of dietary FO in the presence or absence of Indo on hepatic steatosis

Analysis of hepatic lipid profile showed that FO by itself mediated a potent reduction in liver CE and TG, as we reported earlier (9, 27). Indo also mediated a significant reduction in CE and TG accumulation, although to a lesser extent than FO. FO + Indo exerted a more pronounced reduction in CE. The TG-lowering effect of FO in the liver was also moderately increased by Indo. Thus, the CE and TG levels were 30% and 18% lower, respectively, in mice that received FO plus Indo compared with mice that received only FO (Fig. 2A, B). The levels of free cholesterol and free fatty acids were not altered significantly in any of the groups (Fig. 2C, D). Taken together, these data demonstrate that FO reduces hepatic steatosis by reducing CE and TG and that this effect is enhanced by Indo.

Effect of dietary FO in the presence or absence of Indo on hepatic gene profile

Because lipid accumulation was more favorably reduced in mice that received FO + Indo, we next performed a microarray analysis (the microarray data have been deposited in the public genomic data repository [GEO accession number: GSE23742] and are available at <http://www.ncbi.nlm.nih.gov/geo/>) to gain insight into the genes that are up- or down-regulated under this condition. Overall, our data indicated that several genes modulating lipid metabolism, drug and lipid metabolism, and immune response were greatly altered upon combined therapy (Table 2 and supplementary Tables I and II).

Effect of dietary FO in the presence or absence of Indo on genes involved in hepatic lipid metabolism

We performed a real-time PCR analysis to evaluate the microarray data (Fig. 3A–H). The expression of PPAR α -targeted genes such as carnitine palmitoyl transferase-1 and diacyl glycerol O-acyl transferase-1 was increased significantly in mice that received Indo or a combination of FO + Indo. Another PPAR α -induced gene, long-chain acyl coA synthetase-1, was significantly increased only in mice

that received a combination of FO + Indo compared with olive oil-fed control mice. We noted that certain genes involved in peroxisomal lipid metabolism were significantly increased in Indo- and/or FO + Indo-treated mice. For example, the expression of acyl CoA oxidase 1, the key regulatory enzyme of peroxisomal β -oxidation (28), was significantly increased only in mice that received the combination of FO and Indo (1.9-fold; *P* < 0.01). The expression of ATP-binding cassette transporter D3 or the peroxisomal membrane protein 70, which is involved in peroxisomal transport of fatty acids (29), was increased significantly in mice treated with Indo alone (2.6-fold) and in mice treated with FO + Indo (2.9-fold) (*P* < 0.001). Acyl CoA thioesterase-3 (ACOT-3), another gene involved in fatty acid metabolism in peroxisomes, was significantly up-regulated in Indo- and FO + Indo-treated mice. Overall, the expression of PPAR α -targeted genes and peroxisomal genes was increased in Indo- and/or FO + Indo-treated mice, and the combined treatment was more potent in this response. The expression of lipogenic genes, in particular fatty acid synthase (FASN), was significantly decreased in mice treated with Indo (*P* < 0.01) and FO + Indo (*P* < 0.001). The mRNA expression of ACC-1, another gene involved in de novo lipogenesis, was not altered significantly.

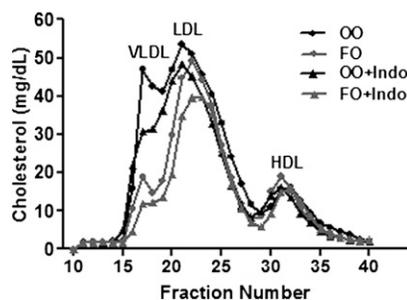


Fig. 1. Lipoprotein distribution in LDLR^{-/-} mice fed a FO diet in the presence or absence of Indo. Plasma lipoproteins were separated by gel filtration chromatography to determine cholesterol distribution. Fractions containing VLDL, LDL, and HDL are indicated on the graph.

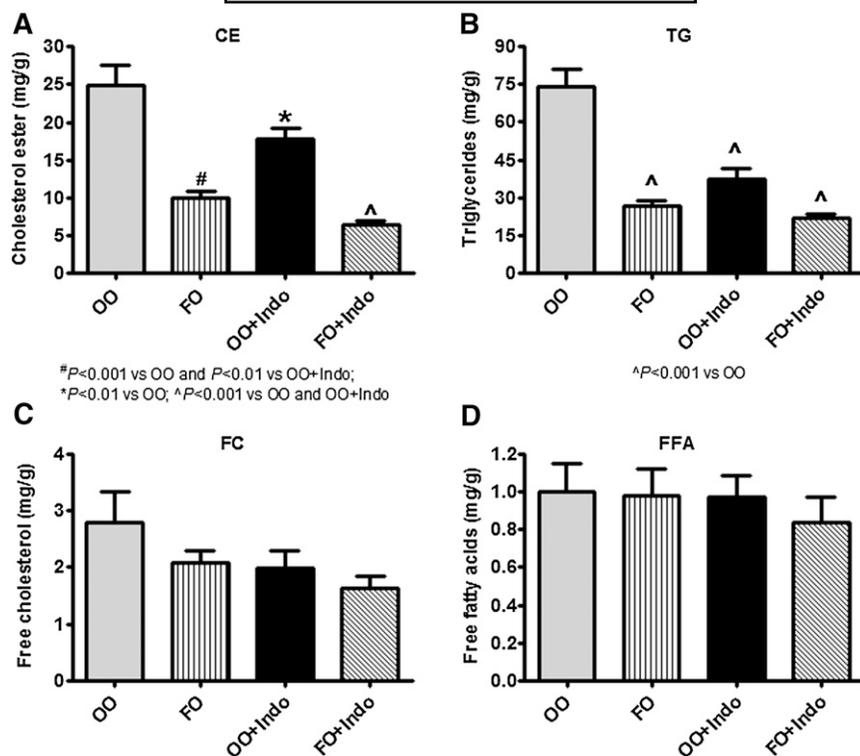


Fig. 2. Effect of FO in the presence or absence of Indo on hepatic steatosis. Hepatic content of CE (A), and TG (B), FC (C), and FFAs (D) were measured by GC (n = 5–10). Data are plotted as mean ± SEM of 5–10 per group.

Effect of dietary FO in the presence or absence of Indo on genes involved in drug and lipid metabolism

Our microarray analysis revealed that various CYPs involved in drug or lipid metabolism were up-regulated in mice that received FO in the presence of Indo (Table 2 and Supplementary Table I). We confirmed the microarray data using real-time PCR analysis, which showed significant 17.6- and 4-fold increases ($P < 0.05$) in the expression of CYP 2C39 and CYP 2B10, respectively, in FO + Indo-treated mice compared with olive oil-fed control mice (Fig. 4A, B). We also analyzed the mRNA expression of one player in bile acid synthesis, CYP 7A1, and noted that its expression was not altered significantly in any of the groups (Fig. 4C). We next wanted to determine the protein levels of CYPs by Western blot analysis. The antibody for CYP2C39 is not commercially available, and therefore we could not verify the protein level of CYP 2C39 in our samples. Western blot analysis of CYP 2B10 showed that this protein is not altered in FO + Indo-treated mice (data not shown). Because our microarray data showed that the PXR gene (Nr1i2) is up-regulated in FO + Indo-treated mice (supplementary Table I), we next analyzed the protein levels

of PXR, a nuclear receptor that regulates the expression of several CYPs of 2C and 3A family. We found that the protein expression of PXR was increased significantly in mice treated with FO and Indo alone. Moreover, PXR level was increased to a greater extent in FO + Indo-treated mice compared with the other groups. Furthermore, in line with the microarray gene expression data, the protein levels of CYP 3A, a downstream target of PXR, were significantly increased in FO + Indo-treated mice. The protein expression of CYP4A, a PPAR α target and another CYP shown to be up-regulated in our microarray data, also increased significantly in mice that received a combination of FO and Indo (Fig. 4D–G). Taken together, these data show that the expression of PXR and several CYPs is greatly increased in mice that received a combination of FO and Indo.

Effect of dietary FO in the presence or absence of Indo on hepatic lipid metabolism and TG secretion rate

Because several PPAR α and PXR target genes were up-regulated in FO + Indo-treated mice, we wanted to determine the activities of PPAR α and PXR in liver samples.

TABLE 2. Number of genes up- or down-regulated in FO-, Indo-, or FO + Indo-treated mice

Biological Function	Number of Genes Up-regulated			Number of Genes Down-regulated		
	FO	Indo	FO + Indo	FO	Indo	FO + Indo
Lipid metabolism	2	22	51	9	2	11
Drug/lipid metabolism (CYPs)	2	3	9	0	0	3
Inflammatory response	1	34	15	41	1	52

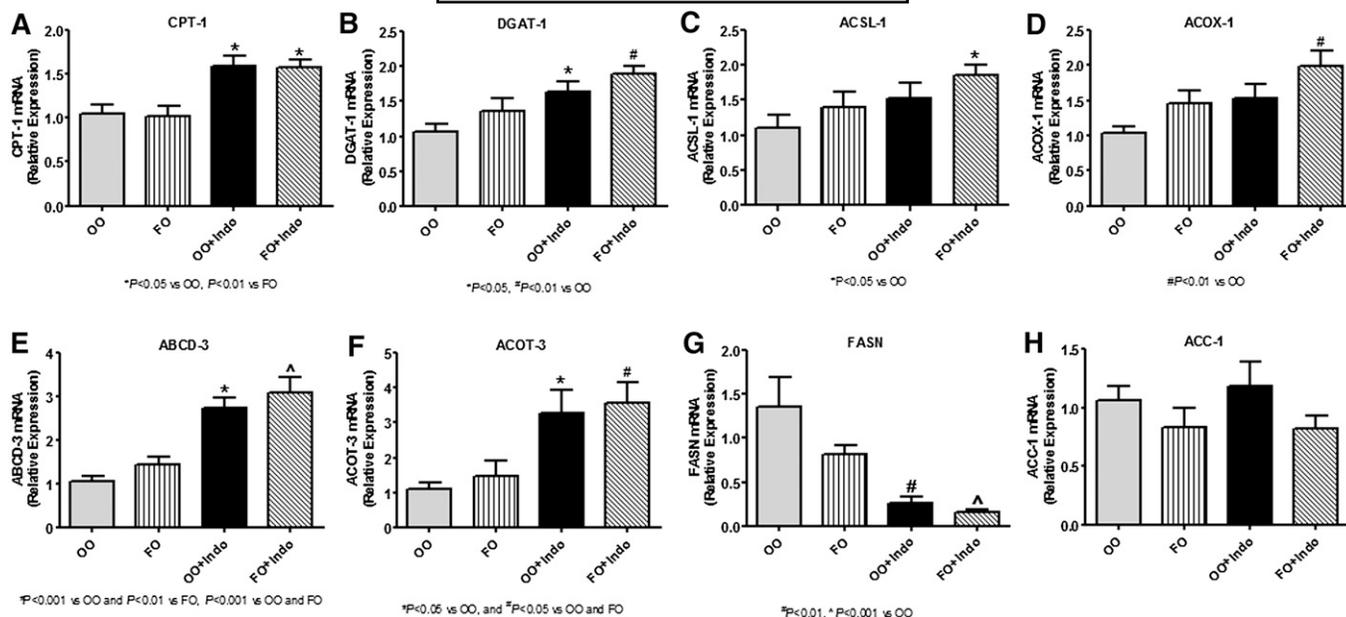


Fig. 3. Effect of FO in the presence or absence of Indo on genes involved in lipid metabolism in liver. RNA samples were analyzed by real-time PCR for the expression of genes modulating lipid metabolism (A–H). Values are expressed as mean \pm SEM of six samples in each group.

Western blot analysis of nuclear extracts revealed that the activities of PPAR α showed only a trend toward an increase in FO- and FO + Indo-treated mice. On the other hand, the nuclear level of PXR was significantly increased in mice that received FO + Indo (Fig. 5A–C). We next analyzed hepatic fatty acid oxidation by incubating liver homogenates in the presence of [14 C] palmitic acid and measuring and summing the levels of [14 C]CO $_2$ and 14 C-labeled acid soluble metabolites. CO $_2$ production is indicative of complete oxidation of fatty acids, whereas acid-soluble metabolites (ASMs) are a measure of the incomplete oxidation of fatty acids. Our data showed that the level of [14 C]CO $_2$ is reduced significantly in FO- and FO + Indo-treated mice. On the other hand, we noted a mild but significant increase in the levels of ASMs in these two groups, suggesting the potential involvement of peroxisomal fatty acid oxidation in these two groups. Nevertheless, the total palmitate oxidation (i.e., the sum of CO $_2$ produced and ASMs), which is a measure of total β -oxidation, was not altered significantly in any of the groups (Fig. 5D–F). Analysis of TG secretion rate revealed that the mice that received both FO and Indo secreted less TG than the control mice (Fig. 5G).

Effect of dietary FO in the presence or absence of Indo on the expression of genes involved in inflammation

We noted that the expression of MCP-1 was decreased significantly in mice that received FO (58%; $P < 0.01$) or Indo (43%; $P < 0.05$) individually. A further decrease in MCP-1 expression was seen in mice that received a combination of FO and Indo (80%; $P < 0.001$) (Fig. 6A). Similarly, the expression of MIP-1 α was significantly reduced in mice that received either FO (65%) or Indo (80%) compared with olive oil-treated control mice ($P < 0.001$). A much greater decrease in MIP-1 α (90%) was noted in mice that received a combination of FO and Indo ($P < 0.001$)

(Fig. 6B). Furthermore, the expression of MMP-12 was reduced significantly in FO- or Indo-treated mice ($P < 0.001$), and MMP-12 expression was almost abolished in mice that received FO + Indo (Fig. 6C). The expression of IL-1 α was decreased significantly in FO- and FO + Indo-treated mice ($P < 0.05$) (Fig. 6D). Although the expression of tumor necrosis factor alpha and serum amyloid A-1 was not altered significantly when compared with olive oil-treated control mice, the expression of these genes in FO + Indo-treated mice was significantly reduced when compared with Indo treatment (Fig. 6E, F).

Effect of dietary fish oil in the presence or absence of Indo on the mRNA expression of genes involved in inflammation or lipid metabolism in white and brown AT

Analysis of the white AT (perigonadal AT) showed that the mice that received the FO diet exhibited a trend toward a decrease in F4/80 and MCP-1, markers of macrophages and inflammation, respectively. However, the levels of these markers were not altered significantly in any of the groups (Fig. 7A, B). Moreover, none of the groups showed a significant difference in the expression levels of F4/80 and MCP-1 in BAT (Fig. 7C, D). To determine whether the enhanced lipid-lowering effect seen with the FO + Indo-treated groups may also be due to increased lipid metabolism in the BAT, we analyzed the mRNA expression of uncoupling protein-1 and PPAR γ coactivator-1 α . We noted that the expression of these two genes was not altered significantly in any of the groups compared with olive oil-fed control mice (Fig. 7E, F).

Effect of dietary FO in the presence or absence of Indo on the levels of COX-derived eicosanoids in liver

TXB $_2$ and PGE $_2$ are considered to be derived mainly from AA via COX-1 and COX-2 activities, respectively. To

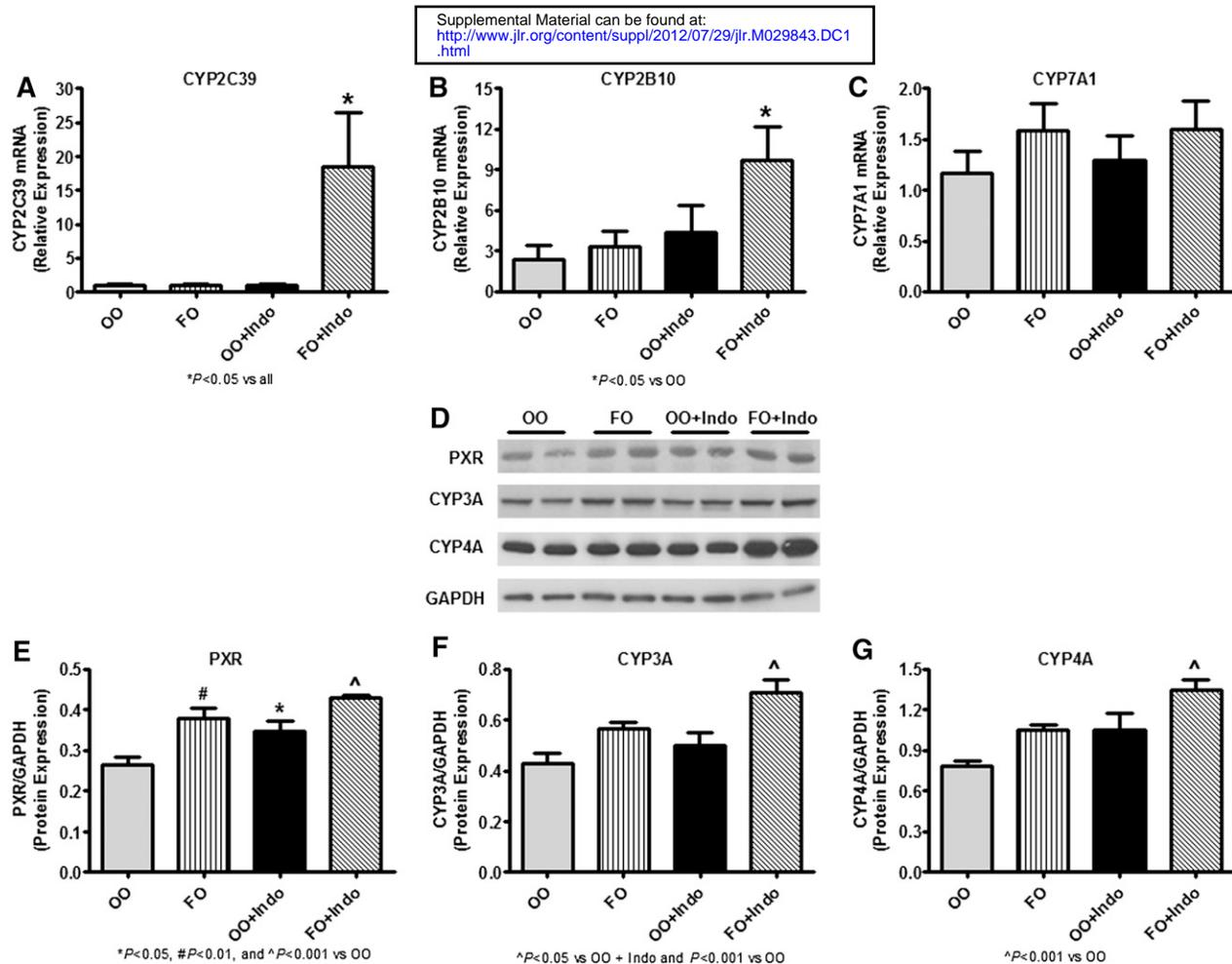


Fig. 4. Effect of FO in the presence or absence of Indo on the mRNA and protein expression of genes involved in lipid or drug metabolism in liver. RNA samples were analyzed by real-time PCR for the expression of genes modulating lipid or drug metabolism (A–C). Values are expressed as mean \pm SEM of six samples in each group. Liver homogenates were analyzed by Western blot analysis for the protein expression of genes modulating lipid or drug metabolism (D–G). Bands from two samples in each group are shown. Values are expressed as mean \pm SEM of five to six samples in each group.

determine whether Indo, an isoform-nonspecific COXIB, inhibits COX activity in our experimental condition, we determined the levels of TXs and PGs. We found that the levels of the AA-derived 2-series eicosanoids, such as TXB₂ and PGE₂, were significantly reduced in the liver of mice that received FO or Indo individually or in combination ($P < 0.001$) (Fig. 8A, D). FO was as potent as Indo in reducing the formation of these metabolites from AA. Because the n-3 fatty acids can also be metabolized by COX activity, it is plausible that the reduction in these 2-series TXs and PGs may be associated with an increase in 3-series metabolites in FO-fed mice. To check this possibility, we measured TXB₃ and PGE₃ in liver samples (Fig. 8B, E). There was a trend toward an increase in TXB₃ and PGE₃ in FO-fed mice, although this effect was not significant. Moreover, the magnitude of the formation of 3-series eicosanoids from FO was much less compared with 2-series metabolites derived from AA. Thus, the cumulative levels of TXB₂+TXB₃ and PGE₂+PGE₃ were much less in FO-fed mice compared with olive oil-fed control mice (Fig. 8C, F). Next, we determined COX activity in the plasma samples. The plasma levels of TXB₂ were significantly reduced in mice receiving either FO or Indo ($P < 0.001$) (Fig. 8G). TXB₂ formation

was nearly abolished in mice that received both FO and Indo. Although FO feeding resulted in a significant increase in TXB₃ levels, the magnitude of TXB₃ production was far less compared with TXB₂ formation from AA in olive oil-fed control mice (Fig. 8H). Thus, the level of TXB₂ in olive oil-fed control mice (Fig. 8G) was 75 ng/ml, and that of TXB₃ in FO-fed mice (Fig. 8H) was 2 ng/ml. The overall effect of FO on COX activity is further evident from the combined levels of 2- and 3-series eicosanoids in plasma. The cumulative levels of these eicosanoids were greatly decreased in FO-fed mice compared with olive oil-fed mice (Fig. 8I). Thus, in addition to proving that COX activity is efficiently blocked by Indo, our data show that the n-3 fatty acids are not preferentially metabolized by COX pathways and that other pathways may be involved in mediating the anti-inflammatory effects of n-3 fatty acids.

DISCUSSION

In the current study, we tested the hypothesis that the lipid-lowering effects of FO can be enhanced by combining with COXIBs, in particular Indo. Although FO is a well-known TG-lowering agent, we and others have shown that it also

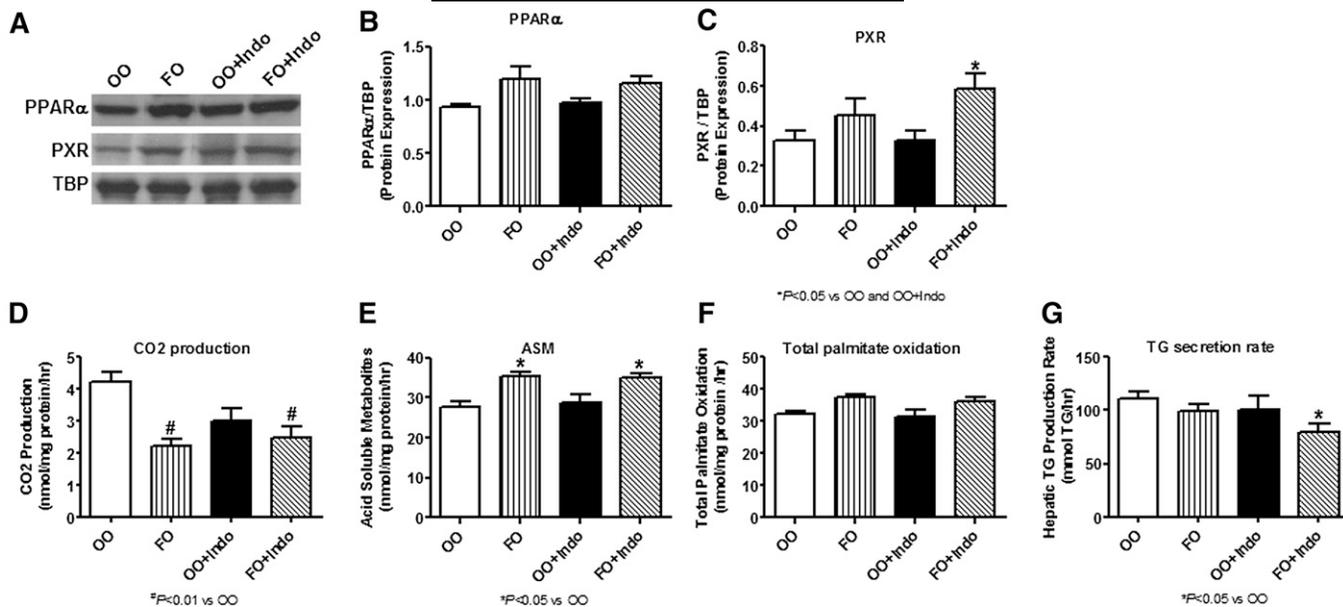


Fig. 5. Effect of FO in the presence or absence of Indo on factors modulating lipid levels in liver and plasma. Nuclear extracts were prepared from liver samples, and Western blot analysis was carried out to detect the activation of PPAR α and PXR (A–C). TATA binding protein was used as a loading control, and a representative band from each group is shown. The rate of β -oxidation was measured using [¹⁴C] palmitic acid (D–F). The TG secretion rate was studied by injecting Triton, a lipoprotein lipase inhibitor, and measuring plasma TG levels over a 3 h period (G). Values are mean \pm SEM of six or seven samples in each group.

reduces plasma cholesterol levels (9, 10). Along these lines, in the present study, we have demonstrated that FO leads to a decrease in TG and TC levels in plasma (Table 1). Indo alone leads to a significant decrease in plasma TC and TG in LDLR^{-/-} mice (Table 1) and a marked reduction in

VLDL cholesterol levels (Fig. 1). We have also shown that the combination of FO and Indo leads to an overall enhancement in the lipid-lowering effect in plasma and liver.

We next studied the potential mechanisms involved in regulating lipid levels in liver and plasma. Because peroxisomes

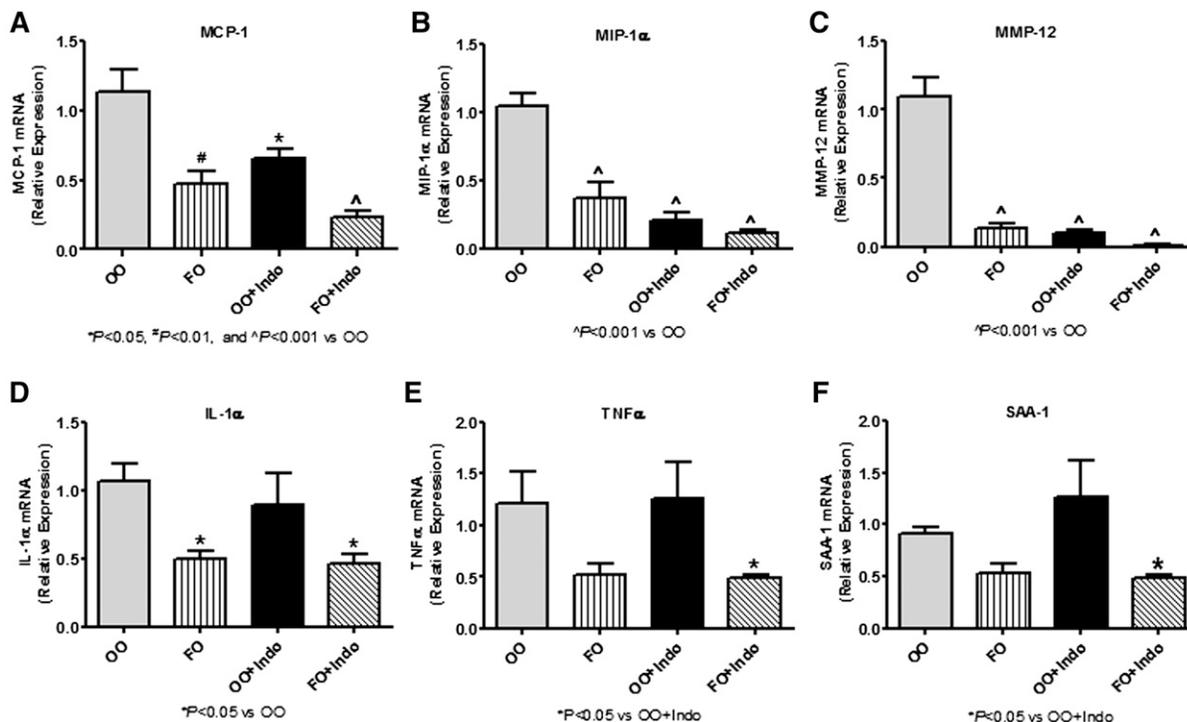


Fig. 6. Effect of FO in the presence or absence of Indo on genes involved in inflammatory response. RNA samples were analyzed by real-time PCR for the expression of genes modulating the inflammatory response (A–F). Values are expressed as mean \pm SEM of six samples in each group.

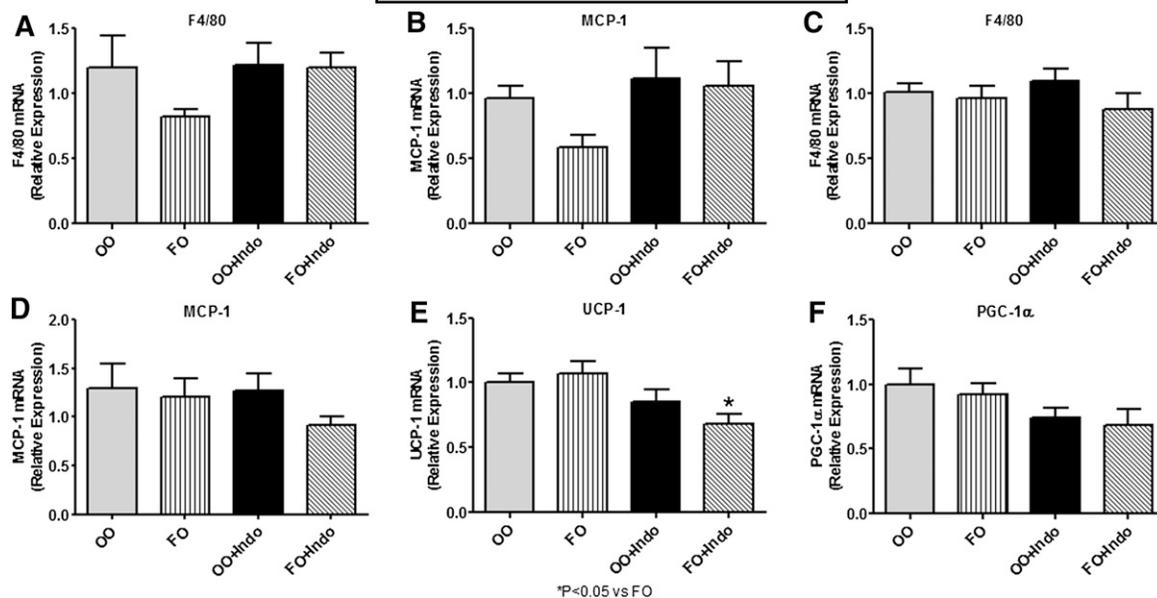


Fig. 7. Effect of FO in the presence or absence of Indo on genes involved in inflammatory response and/or lipid metabolism in perigonadal AT and BAT. RNA samples were analyzed by real-time PCR for the expression of genes modulating the inflammatory response in perigonadal AT (A and B) and inflammatory response and lipid metabolism BAT (C–F). Values are mean \pm SEM of six or seven samples in each group.

play an important role in modulating hepatic lipid metabolism and subsequent nonalcoholic fatty liver disease (as reviewed in Ref. 30), we analyzed the expression of certain genes involved in peroxisomal lipid metabolism and found that the expression of ABCD-3 and ACOT-3 is significantly increased in Indo-treated mice. Moreover, mice treated with both FO and Indo exhibited a greater increase in the expression of ABCD-3 and ACOT-3. In addition, the expression of acyl CoA oxidase 1, another peroxisomal gene, was significantly increased only in FO + Indo-treated mice, suggesting that the combined treatment is more potent in activating the peroxisomal pathways. We next analyzed the nuclear extracts for the level of PPAR α and found no significant increase in any of the groups compared with control. Analysis of hepatic fatty acid oxidation showed that levels of CO₂ were reduced significantly in FO and FO + Indo groups, whereas those of ASMs were increased significantly. Because the ASMs represent the products derived from incomplete oxidation of fatty acids and because peroxisomal pathways result in incomplete oxidation products of fatty acids, our data suggest that the peroxisomal lipid metabolism may be increased to some extent in the FO- and FO + Indo-treated groups. However, this effect was not greater in FO + Indo-treated mice compared with mice treated with FO alone. Therefore, the role of peroxisomal pathways in mediating the enhanced lipid-lowering effect of the combination of FO and Indo is unclear. Analysis of TG secretion rate showed that the mice that received both FO and Indo displayed a significant decrease in TG secretion rate compared with control mice, suggesting that the enhanced plasma lipid-lowering effect of this combination may be due to reduced VLDL secretion into the plasma. The mRNA expression of FASN was potently reduced in Indo- and FO + Indo-treated mice. Although the protein

level of FASN is not altered by Indo treatment, it was decreased by combined treatment with FO and Indo (data not shown).

We next wanted to study the potential biochemical mechanisms by which this combination is beneficial. COXs have been implicated in partly mediating the anti-inflammatory effects of n-3 fatty acids (17). For example, it has been reported that COX-derived ω -3 fatty acid metabolites, such as 3-series TXs and PGs, are anti-inflammatory or less pro-inflammatory compared with the 2-series eicosanoid metabolites derived from AA (17). However, it has been reported that eicosapentaenoic acid is a relatively poor substrate for COX (31); moreover, docosahexaenoic acid, another n-3 fatty acid found in FO, has been shown to be a strong inhibitor of PG synthesis (32). We found that FO feeding greatly reduced AA-derived 2-series TX and PG formation, which was not associated with an appreciable increase in 3-series eicosanoids in our study. Furthermore, the plasma levels of TXB₂ and TXB₃ in FO-fed mice showed that although the n-3 fatty acids can be metabolized by COX to some extent, it is not their preferred pathway. While the role of COX in mediating the anti-inflammatory effect of FO is still in debate, our data show that FO in the absence of COX activity exhibits a more potent anti-inflammatory effect, indicating that COX-derived metabolites of n-3 fatty acids may not have a significant role in mediating the anti-inflammatory effect of FO. Moreover, these data confirm that COX activity is potently inhibited by Indo in vivo. The levels of these eicosanoid metabolites correlate not only with the anti-inflammatory effects of the combination of FO and Indo but also with their lipid-lowering effects. Although the direct effect of these eicosanoids in modulating lipid metabolism is unclear, our data indicate that the enhanced anti-inflammatory effect of this

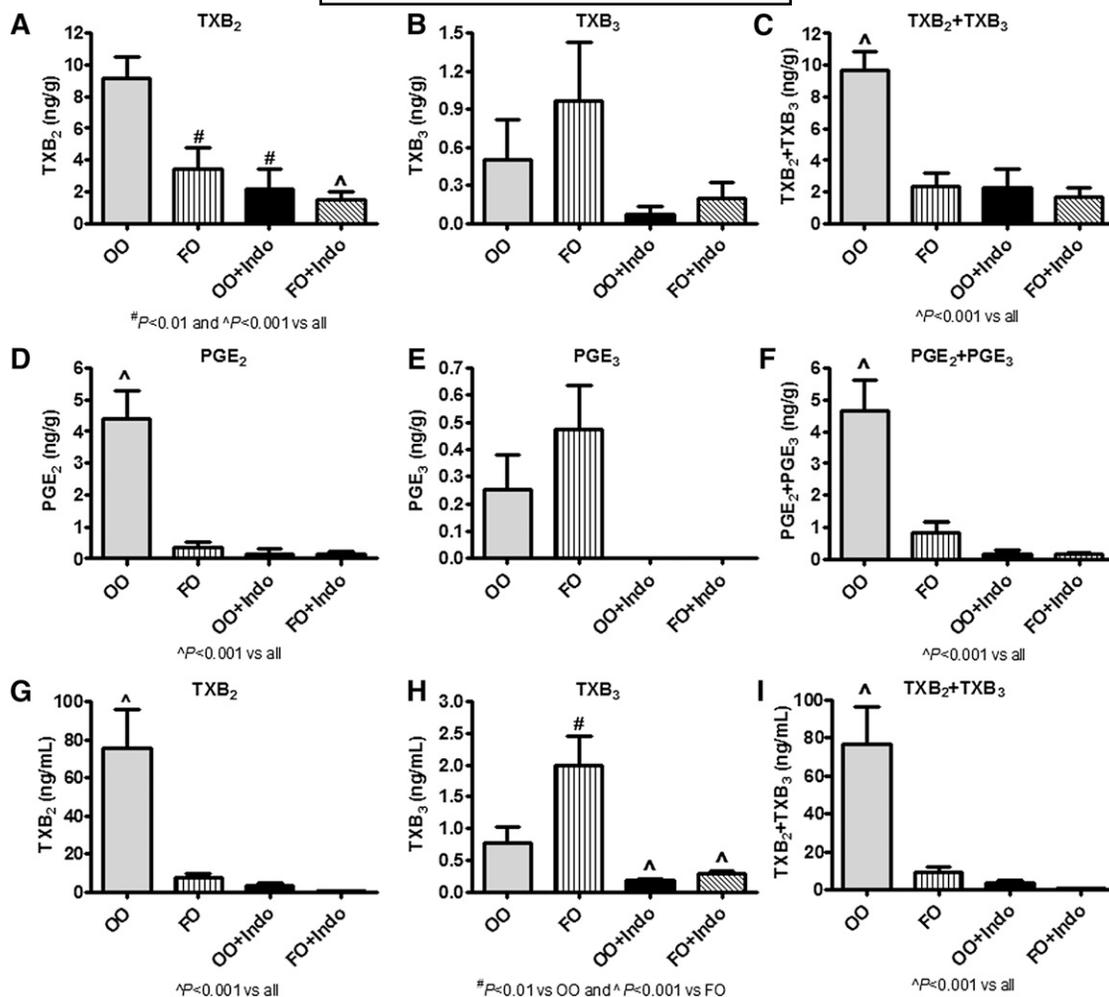


Fig. 8. Effect of FO in the presence or absence of Indo on the levels of various COX-derived eicosanoids in liver and plasma. Liver samples were analyzed for the levels of 2- and 3-series PGs and TXs by mass spectrometry (A–F). Values are expressed as mean \pm SEM of six samples in each group. Ionomycin-induced TXB₂ formation in blood was measured by mass spectrometry (G–I). Values are mean \pm SEM of 9–14 samples in each group.

combination may have a role in potentiating the lipid-lowering effects.

Although modulation of AA metabolism is critical to modulating the inflammatory response by FO or COX-IBs, AA is metabolized not only via COX activity but also through several other pathways. For example, AA can be metabolized by lipoxygenases and cytochrome P450s, giving rise to products that could modulate inflammation. For example, Serhan et al. (19) have shown that certain lipoxygenase-derived lipid mediators of AA, the lipoxins, are anti-inflammatory and thus play a role in the resolution of inflammatory response (as reviewed in Ref. 33). They have also shown that the n-3 fatty acids can produce potent anti-inflammatory agents, termed “resolvins,” via lipoxygenase activity (33). The ω -3 fatty acids can be converted to 5-series leukotrienes via lipoxygenase activity, and these are known to be less inflammatory compared with the 4-series leukotrienes that are derived from AA. Therefore, the role of the lipid derivatives produced by these enzymes in mediating the enhanced anti-inflammatory effect of the combination of FO and Indo remains possible.

Regarding the CYP pathways, AA can be metabolized by the CYP2C family to epoxy eicosatrienoic acid, which is a potent anti-inflammatory mediator (34) and a strong PPAR agonist (35). Moreover, it has been recently shown that such epoxy derivatives can be produced from n-3 fatty acids (36, 37). The expression of CYP2C39, which is known to produce eicosatrienoic acid (38), is significantly increased in mice that received both FO and Indo. Thus, our data indicate that the enhanced CYP-mediated metabolism of n-3 fatty acids may play a role in the synergistic anti-inflammatory effect of this combination.

In addition to modulating the inflammatory response, the CYPs play an important role in regulating fatty acid and cholesterol metabolism. For example, CYP4A, a downstream target of PPAR α , is the major regulator of fatty acid ω -oxidation (39), and our data show that the protein level of CYP 4A is significantly increased upon combined treatment with FO and Indo. Our data also show that the expression of PXR and its target gene CYP3A were increased to a greater extent in FO + Indo-fed mice compared with other groups. Furthermore, we provide evidence that the activation of PXR, as measured by the nuclear levels of this

protein, is significantly increased in FO + Indo group compared with control. PXR was originally identified as a xenobiotic sensing nuclear receptor playing a role in drug metabolism, and it is now well known to play a role in bile acid metabolism (40). CYP 3A, a PXR target, is known to regulate drug and bile acid detoxification, and recently it has been shown to promote cholesterol catabolism by converting cholesterol to 27-hydroxycholesterol (41). Thus, it is reasonable to speculate that the improved cholesterol metabolism in FO + Indo-treated mice is mediated through PXR and CYPs. In fact, based on their role in regulating cholesterol homeostasis, it has been postulated that CYPs may serve as the therapeutic targets for cholesterol-lowering drugs (42), and our data support this notion. Although our data point to the role of PXR in mediating the hypolipidemic effects of this combination, the role of constitutive androstane receptor, another nuclear receptor that acts in coordination with PXR, cannot be ruled out.

Taken together, our data that FO increases adiposity, the formation of ASMs in liver, and the expression of PXR suggest that the improved lipid storage in adipose tissue and the increased lipid catabolism in liver play a role in reducing the fasting FFA levels in plasma and/or the overall lipid-lowering effect of FO. Although FO is a well-known lipid-lowering agent, the effect of Indo in modulating lipid metabolism has not been clearly elucidated. As for hepatic steatosis, Indo was shown to increase hepatic lipid metabolism and to prevent hepatic lipid accumulation in a mouse model of cancer (43). Our data show that Indo blunts the accumulation of CE and TG in the liver. This effect is associated with a significant decrease in plasma TC and TG in LDLR^{-/-} mice (Table 1).

Regarding potential mechanisms, our data show that Indo-treated mice exhibit an increased mRNA expression of peroxisomal genes such as ABCD-3 and ACOT-3. On the other hand, the fatty acid β -oxidation measured using [1-¹⁴C]palmitic acid is not altered in these mice. However, in vivo peroxisomal metabolism of fatty acids leads to chain shortening but not to complete fatty acid oxidation. Moreover, we only used palmitic acid as a substrate for β -oxidation; therefore, the condition does not exactly mimic the in vivo conditions. Therefore, it is reasonable to speculate that the peroxisomal pathways may be involved in mediating the lipid-lowering effect of Indo. Furthermore, the protein expression of PXR is increased upon Indo treatment. Because PXR is involved in drug and cholesterol metabolism, it is possible that PXR may also have a role in mediating the hypolipidemic effect of Indo. Further studies are warranted to determine the specific role of peroxisomal fatty acid oxidation and PXR in mediating the hypolipidemic effects of Indo.

Although combination therapy using different drugs is frequently used in clinical practice, our data suggest that combining FO with Indo may be an effective strategy to combat dyslipidemia and hepatic steatosis. Although several beneficial effects are noted in the current study, the plasma TC and liver CE were reduced by an additional 21% and 30%, respectively, in mice that received a combination of FO and Indo compared with mice that received

FO only. Moreover, unlike statins, which reduce only plasma TC (44), this combination appears to be beneficial in reducing TC and TG. Furthermore, we noted an overall decrease in hepatic inflammation in mice that received a combination of FO and Indo, further indicating the safety of this combination. Our experimental diet contained 6% fish oil, which provides twice the maximum dose (4 g/day) of n-3 fatty acids recommended in clinical settings. To better understand the efficacy of combination therapy, further studies are needed using lower amounts of fish oil in the diet.

In summary, our data suggest that a combination of FO and Indo mediates enhanced lipid-lowering effects in plasma and liver via several mechanisms, including increased lipid accumulation in perigonadal fat, increased hepatic lipid metabolism via PXR and CYP activities, and reduced TG secretion from liver. However, the mechanisms by which Indo mediates its lipid-lowering effects are unclear, and future studies are needed to determine the exact mechanisms by which Indo, an anti-inflammatory agent, exerts its hypolipidemic effect. Our findings have implications in considering a combination of FO and COXIBs as a potential therapeutic regimen to treat patients with dyslipidemia, and in particular patients with FH, who are at an increased risk of developing premature cardiovascular disease. **■**

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