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Fish Oil and Indomethacin in Combination Potently Reduce Dyslipidemia and Hepatic Steatosis in LDLR-/- Mice

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Running Title: Fish oil and indomethacin potently reduce dyslipidemia

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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, 6mg/liter 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 to control. The mRNA and/or protein expression of pregnane X receptor (PXR) and cytochrome P450 (CYP 450) isoforms that alter inflammation and/or lipid metabolism are increased to a greater extent in mice that received FO + Indo. Moroever, 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 CYP 450s.

Supplementary key words: n-3 fatty acids, cytochrome P450s, COXIBs, PXR, cholesterol, triglycerides

INTRODUCTION

Dyslipidemia characterized by elevated cholesterol or triglycerides is a major risk factor for coronary heart disease (CHD) (reviewed in (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 approximately 1 in 500 people and can cause premature atherosclerotic disease (Reviewed in (2)). Combination therapy is being commonly employed 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 statin-induced side effects like muscle toxicity limit the use of this combination (Reviewed in (3, 4)). In addition to dyslipidemia, inflammation by itself can lead to increased risk of CHD. Therefore, a therapeutic regimen that has the potential to reduce both dyslipidemia and inflammation would be more beneficial in reducing cardiovascular events than treatments aimed at improving dyslipidemia alone.

Fish oil (FO) containing the n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is a potent anti-inflammatory (reviewed in (5)) and triglyceride (TG) lowering agent (6). In addition to its TG lowering effect, FO has also been reported to reduce LDL cholesterol levels in both FH and normal subjects (7). Case reports have shown that FO may be beneficial to children having 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 only 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 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). Futhermore, several anti-inflammatory agents have been shown to promote hepatic lipid metabolism. For example, dietary components such as curcumin, baicalin and capsaicin with known anti-inflammatory properties have been shown to ameliorate metabolic disorders and hepatic steatosis (13-15). With regard to 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 both 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 pro-inflammatory. Alternatively, the n-3 fatty acids replace AA from the membrane phospholipids thereby reducing the availability of AA to COX thus reducing the production of 2-series eicosanoids from AA. It should also be noted that the n-3 fatty acids by themselves can be metabolized by COX giving rise to 3-series eicosanoids that are anti-inflammatory or less pro-inflammatory compared to AA-derived 2-series eicosanoids (17). Overall, the n-3 fatty acids and 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 cyclooxygenase inhibitors (COXIBs) may exert an enhanced anti-inflammatory effects. In fact, a synergistic anti-inflammatory effect of this combination has been reported in monocytes (18). Moreover, Serhan *et al* have shown that aspirin, a COX inhibitor, triggers the formation of potent anti-inflammatory agents called resolvins from n-3 fatty acids (19). 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 non-specific 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 fish oil in alleviating dyslipidemia and hepatic steatosis in hyperlipidemic patients who are at an increased risk of premature CVD mortality.

MATERIALS AND METHODS

Mice: LDLR-/- mice originally obtained from Jackson Laboratories were used in the present study. Two to three month old female LDLR-/- mice 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 (6mg/liter) (20) in drinking water. A stock of Indo was made in DMSO which is then mixed in drinking water. The water containing Indo was changed every day. The olive oil, 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 2 cages of mice per group. At the end of 12 wk, the mice were fasted for 5 h and sacrificed 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.

Plasma measurements: Plasma total cholesterol (TC) and triglycerides (TG) were analyzed using kits from Raichem. Plasma free fatty acids (FFA) were measured using kits from Wako chemicals. Plasma levels of cholesterol ester (CE), free cholesterol (FC) and phospholipids (PL) were analyzed by gas chromatography (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 (μU/mL) × fasting serum glucose (mg/dL)/405. Plasma lipoprotein profiles were analyzed by fast protein liquid chromatography (FPLC) using superose 6 column (Amersham Pharmacia). One hundred μl of pooled plasma samples were loaded onto the column and forty 0.5 ml fractions were collected for cholesterol measurement. Fractions 15-20 contain VLDL, 21-26, LDL and 27-34, HDL.

Lipid analysis: The levels of cholesterol ester (CE) and TG in liver samples were determined by gas chromatography at the Lipid Core Laboratory of Vanderbilt University.

Microarray analysis: The microarray analysis of liver RNA samples were carried out using pooled RNA samples (6 samples per group) at the Functional Genomics Shared Resouces at Vanderbilt University. Further details for the experimental procedures are provided in the online supplemental data.

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 ΔΔCT method to quantify the mRNA expression levels. Except for low expression genes (inflammatory genes), we have 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 EDTA, 1 mmol/L EGTA, 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 3A44 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 PPARα and PXR using anti-PPARα and anti-PXR antibodies, respectively (Santacruz Biotech). We used TATA binding protein (TBP) as a loading control and anti-TBP antibody was purchased from ABCAM.

Measures of fatty acid oxidation. Fatty acid oxidation was measured in whole liver homogenates by measuring and summing $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of [1-^{14}C]-palmitic acid as previously described (22-24).

Triglyceride secretion rate: The TG secretion rate was measured as described earlier (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, purchased from Sigma, at a concentration of 500 mg/kg body weight. The blood samples were collected from tail vein every 1 h up 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 E_2 (PGE_2), prostaglandin E_3 (PGE_3), thromboxane B_2 (TXB_2), and thromboxane B_3 (TXB_3) in liver were analyzed by gas chromatography (GC)/mass spectrometry (MS) at the Eicosanoid Core Laboratory of Vanderbilt University. Please see the online supplemental data for details of this experiment.

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 sacrifice were stimulated with ionomycin (50 µM) for 30 min at 37°C and centrifuged at 3000 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 online-supplemental data.

Statistical Analysis: Values are presented as the mean ± 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 to control. The cumulative food intake was not altered much among different groups. The fat mass as measured by NMR trended towards an increase in FO and FO + Indo groups. However, the adiposity (% body weight) showed a significant increase in FO fed mice compared to 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 towards 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 to olive oil fed control mice. Interestingly, 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 to mice that received only the FO diet ($P<0.05$). The CE level showed only a trend towards a decrease in FO fed mice. However, a significant reduction in CE was noted in mice that

received FO + Indo compared to olive oil controls. Thus mice receiving both FO and Indo showed more than 20% reduction in plasma TC and CE compared to mice that received FO only. Plasma TG levels were decreased significantly in FO ($P<0.001$), Indo ($P<0.01$), and FO + Indo ($P<0.001$) treated 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 while FO by itself exerts a potent cholesterol lowering effect in plasma, this effect is potentiated by Indo in LDLR-/- mice. In addition, analysis of lipoprotein profile revealed that FO reduced both VLDL, and LDL cholesterol levels in LDLR-/- mice. It is interesting to note that 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 (Figure 1).

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). Interestingly, Indo also mediated a significant reduction in both CE and TG accumulation however, to a lesser extent than FO. Of note, 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 to 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 both CE and TG and 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 (Microarray data GEO accession number: GSE23742)* to gain insight into the genes that are up or downregulated under this condition. Overall, our data indicated that several genes modulating lipid metabolism, drug/lipid metabolism, and immune response were greatly altered upon combined therapy (Table 2 and Supplemental Tables 1 and 2).

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 PPARα-targeted genes such as carnitine palmitoyl transferase-1 (CPT-1) and diacyl glycerol O-acyl transferase-1 (DGAT-1) was increased significantly in mice that received either Indo or a combination of FO + Indo. Another PPARα-induced gene, long chain acyl coA synthetase-1 (ACSL-1), was significantly increased only in mice that received a combination of FO + Indo compared to olive oil-fed controls. Importantly, 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 (ACOX-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 (ABCD3) or the peroxisomal membrane protein 70 (PMP 70) which is involved in peroxisomal transport of fatty acids (29), was increased significantly in both Indo alone (2.6 fold) and FO + Indo (2.9 fold) treated mice ($P<0.001$). Acyl CoA thioesterase-3 (ACOT-3), another gene involved in fatty acid metabolism in peroxisomes, was significantly upregulated in Indo and FO + Indo treated mice. Overall, the expression of PPARα-targeted genes and peroxisomal genes was increased in Indo and/or FO + Indo treated mice and the combined treatment is more potent in this response. Of note, the expression of lipogenic genes, in particular, FASN is significantly decreased in

Indo ($P<0.01$) and FO + Indo ($P<0.001$) treated mice. The mRNA expression of ACC-1, another gene involved in *de novo* lipogenesis was not altered significantly.

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

Our microarray analysis revealed that various CYPs involved in drug or lipid metabolism were upregulated in mice that received FO in the presence of Indo (Table 2 and Supplemental Table 1). We were able to confirm the microarray data using real-time PCR analysis which showed a significant 17.6- and 4-fold increase ($P<0.05$) in the expression of CYP 2C39 and CYP 2B10, respectively, in FO + Indo received mice compared to olive oil-fed control (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 PXR gene (Nr1i2) is upregulated in FO + Indo treated mice (Supplemental Table 1), 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 FO and Indo alone treated mice. Moreover, PXR level was increased to a greater extent in FO + Indo treated group compared to the other groups. Furthermore, in line with the microarray gene expression data, the protein levels of CYP 3A, a downstream target of PXR was significantly increased in FO + Indo treated mice. The protein expression of CYP4A, a PPARα target and another CYP shown to be upregulated 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 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 PPARα and PXR target genes were upregulated in FO + Indo treated mice, we wanted to determine the activities of PPARα and PXR in liver samples. Western blot analysis of nuclear extracts revealed that the activities of PPARα showed only a trend towards 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 actually reduced significantly in FO and FO + Indo groups. 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, 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 secrete 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. Notably, an even 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-1α was significantly reduced in mice that received either FO (65%) or Indo (80%) compared to olive oil supplemented control ($P<0.001$). A much greater decrease in MIP-1α (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 received mice ($P<0.001$) and MMP-12

expression was almost abolished in mice that received FO + Indo (Fig. 6C). The expression of IL-1α was decreased significantly in both FO and FO + Indo received mice ($P<0.05$, Fig. 6D). Although the expression of tumor necrosis factor (TNFα), and serum amyloid A-1 (SAA-1), was not altered significantly when compared to olive oil control, the expression of these genes in FO + Indo treated mice was significantly reduced when compared to 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 towards 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 as well (7 C&D). To determine whether the enhanced lipid-lowering effect seen with FO + Indo treated groups may also be due to increased lipid metabolism in the BAT, we analyzed the mRNA expression of uncoupling protein-1 (UCP-1) and PPARγ coactivator-1α (PGC-1α). We noted that the expression of these two genes was not altered significantly in any of the groups compared to olive oil-fed control (Fig. 7 E&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 -2 activities, respectively. To determine whether Indo, an isoform non-specific COXIB, does inhibit COX activity in our experimental condition, we next determined the levels of TXs and PGs. We found that the levels of the AA-derived 2-series eicosanoids such as TXB_2 and PGE_2 were significantly reduced in the liver of mice that received FO or Indo individually or in combination (Fig. 8A&D, $P<0.001$). It is interesting to note that 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 also measured TXB_3 and PGE_3 in liver samples (Fig. 8B&E). There was a trend towards an increase in TXB_3 and PGE_3 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 to 2-series metabolites derived from AA. Thus, the cumulative levels of TXB_2+TXB_3 and PGE_2+PGE_3 were much less in FO fed mice compared to olive oil fed control (Fig. 8C&F). Next, we determined COX activity in the plasma samples. The plasma levels of TXB_2 were significantly reduced in mice receiving either FO or Indo (Fig. 8G, $P<0.001$). TXB_2 formation was nearly abolished in mice that received both FO and Indo. Although FO feeding resulted in a significant increase in TXB_3 levels, the magnitude of TXB_3 production was far less compared to TXB_2 formation from AA in olive oil control (Fig. 8H). Thus, the level of TXB_2 in olive oil fed control mice (Fig. 8G) was 75 ng/ml and that of TXB_3 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. As noted in Fig. 8I, the cumulative levels of these eicosanoids were greatly decreased in FO fed mice compared to olive oil fed mice. 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. While FO is a well-known TG-lowering agent, we and others have shown that it also reduces plasma cholesterol levels (9, 10). Along the lines, in the present study, we have demonstrated that FO leads to a remarkable decrease in both TG and TC levels in plasma (Table 1). It is interesting to note that 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 (Figure 1). We have also

shown that the combination of FO and Indo leads to an overall enhancement in the lipid-lowering effect in both plasma and liver.

We next studied the potential mechanisms involved in regulating lipid levels in liver and plasma. Because peroxisomes play an important role in modulating hepatic lipid metabolism and subsequent non-alcoholic fatty liver disease (reviewed in (30)), we analyzed the expression of certain genes involved in peroxisomal lipid metabolism and we 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 ACOX-1, another peroxisomal gene is 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 PPARα and there was no significant increase in any of the groups compared to control. Analysis of hepatic fatty acid oxidation showed that levels of CO_2 were reduced significantly whereas those of ASMs were increased significantly in FO and FO + Indo groups. 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 FO and FO + Indo treated groups. However, this effect was not greater in FO + Indo treated mice compared to FO alone fed mice. 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 to control suggesting that the enhanced plasma lipid-lowering effect of this combination may be due to reduced VLDL secretion into the plasma. Of note, the mRNA expression of FASN was potently reduced in Indo and FO + Indo treated mice. While 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 to the 2-series eicosanoid metabolites derived from AA (17). However, it has been reported that EPA is a relatively poor substrate for COX (31) and moreover DHA, 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 TXB_2 and TXB_3 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 indeed potently inhibited by Indo *in vivo*. It is interesting to note that the levels of these eicosanoid metabolites not only correlate 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 combination may have a role in potentiating the lipid-lowering effects.

While modulation of AA metabolism is critical to modulating the inflammatory response by FO or COXIBs, it should be noted that 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 which could modulate inflammation. For example, Serhan *et al* 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 (reviewed in(33)). They have also shown that the n-3 fatty acids can produce potent anti-inflammatory agents termed resolvins via lipoxygenase activity (33). It should also be pointed out that the ω-3 fatty acids can be converted to 5-series leukotrienes via lipoxygenase

activity and these are known to be less inflammatory compared to 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.

As for the CYP pathways, AA can be metabolized by the CYP2C family to epoxy eicosatrienoic acid (EET) 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 also be produced from n-3 fatty acids (36, 37). It is interesting to note that the expression of CYP2C39 which is known to produce EETs (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.

It should also be pointed out that 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 PPARα 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 to 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 to control. PXR is 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 also 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 (CAR), another nuclear receptor which acts in co-ordination 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 free fatty acid levels in plasma and/or the overall lipid-lowering effect of FO. While FO is a well-known lipid-lowering agent, the effect of Indo in modulating lipid metabolism has not been clearly elucidated. Pratico *et al* have shown that Indo treatment for 18 weeks led to a mild reduction in plasma TC in LDLR-/- mice (20). In line with this, our data provide evidence for the role for NSAIDs, like Indo in improving plasma lipid and lipoprotein profiles in dyslipidemic mice. As for hepatic steatosis, Indo was shown to increase hepatic lipid metabolism and prevent hepatic lipid accumulation in a mouse model of cancer (43). Our data show that Indo blunts the accumulation of both CE and TG in the liver. This effect is associated with a significant decrease in plasma TC and TG in LDLR-/- mice (Table 1).

With regard to 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-^{14}C]-palmitic acid is not altered in these mice. However, it should be noted that *in vivo* peroxisomal metabolism of fatty acids leads only to chain shortening but not in complete fatty acid oxidation. Moreover, we only used palmitic acid as a substrate for β-oxidation and therefore, the condition is not exactly mimicking the *in vivo* conditions. Therefore, it is reasonable to speculate that the peroxisomal pathways may still be involved in mediating the lipid-lowering effect of Indo. It should also be pointed out that the protein expression of PXR is increased upon Indo treatment. As PXR is involved in both drug and cholesterol metabolism, it is possible that PXR may also have a role in mediating the

hypolipidemic effect of Indo. However, further studies are warranted to determine the specific role of peroxisomal fatty acid oxidation and PXR in mediating the hypolipidemic effects of Indo.

While combination therapy using different drugs is frequently employed in clinical practice, our data suggest that combining FO with Indo may be an effective strategy to combat dyslipidemia and hepatic steatosis. While several beneficial effects are noted in the current study, it should be pointed out that 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 to mice that received FO only. Moreover, unlike statins which reduce only plasma TC (44), this combination appears to be beneficial in reducing both 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. It should also be pointed out that our experimental diet contained 6% fish oil which provides twice as much the maximum dose (4g per day) of n-3 fatty acids as recommended in clinical settings. To better understand the efficacy of combination therapy further studies are needed using lower amount 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 still 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, FH, who are at an increased risk of developing premature CVD.

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FOOTNOTES

*The microarray data have been deposited in the public genomic data repository (GEO accession number: GSE23742). These data are available at http://www.ncbi.nlm.nih.gov/geo).

FIGURE LEGENDS

Figure 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 graph.

Figure 2: Effect of FO in the presence or absence of Indo on hepatic steatosis

Hepatic content of cholesterol esters (A), and triglycerides (B), free cholesterol (C), and free fatty acids (D) were measured by gas chromatography (n=5-10). Data are plotted as mean ± SEM of 5-10 per group. Cholesterol ester (CE), Triglycerides (TG), free cholesterol (FC), and free fatty acids (FFA).

Figure 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 ± SEM of six samples in each group.

Figure 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 ± 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 ± SEM of six samples in each group.

Figure 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 PPARα and PXR (A-C). TBP was used as a loading control and a representative band from each group is shown. The rate of β-oxidation was measured using [^{14}C] 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 ± SEM of 6-7 samples in each group.

Figure 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 ± SEM of six samples in each group.

Figure 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&B) and, inflammatory response and lipid metabolism BAT (C-F). Values are mean ± SEM of 6-7 samples in each group.

Figure 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 ± SEM of six samples in each group. Ionomycin induced TXB_2 formation in blood is measured by mass spectrometry (G-I). Values are mean ± SEM of 9-14 samples in each group.

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
Total Cholesterol (TC, mg/dL)	572 ± 21	393 ± 24[b]	469 ± 19[a]	311 ± 19[bc]
Cholesterol Ester (CE, mg/dL)	638 ± 43	489 ± 38	651 ± 42	378 ± 35[a]
Unesterified Cholesterol (UC,	210 ± 23	150 ± 9.7	194 ± 37	105 ± 11.7

mg/dL)				
Triglycerides (TG, mg/dL)	209 ± 18	84 ± 8[b]	151 ± 12[b]	87 ± 8[b]
Phospholipids (PL, mg/dL)	386 ± 23	363 ± 56	341 ± 22	264 ± 23
Free Fatty Acids (FFA, 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]$P<0.001$, vs olive oil; [c]$P<0.05$ vs FO; [d]$P<0.05$ vs 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 PL. Food intake was measured in two cages in each group and CE, UC, and PL were analyzed using GC method and values are mean ± SEM of 4-6 samples in each group.

Table 2: Number of genes up or downregulated in FO, Indo, or FO + Indo received mice

Biological Function	Number of genes upregulated			Number of genes downregulated		
	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

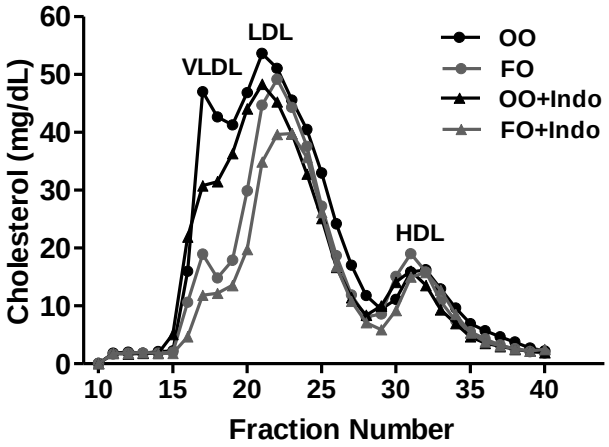

60
50
40
30
20
10
0
Cholesterol (mg/dL)
10
15
20
25
30
35
40
Fraction Number
VLDL
LDL
HDL
OO
FO
OO+Indo
FO+Indo

Figure 1

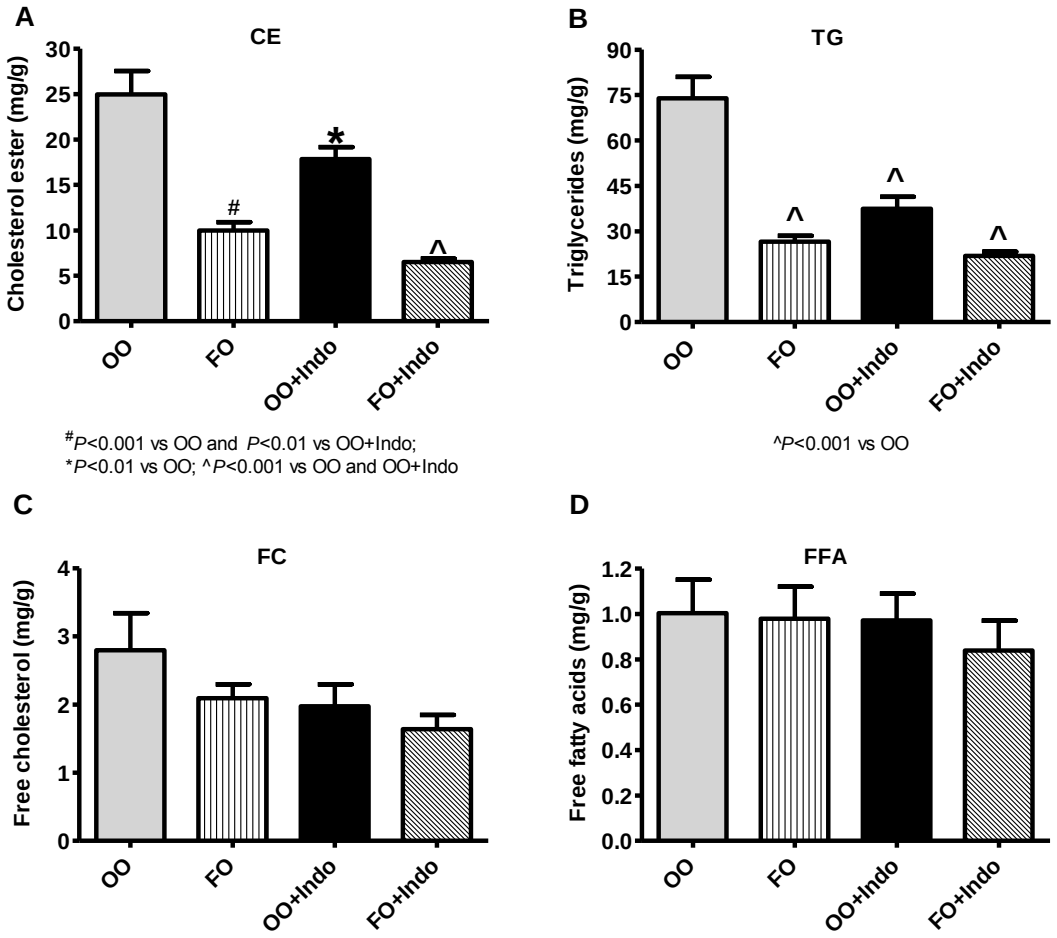

A
CE
Cholesterol ester (mg/g)
30
25
20
15
10
5
0
#
*
^
OO
FO
OO+Indo
FO+Indo
#P<0.001 vs OO and P<0.01 vs OO+Indo;
*P<0.01 vs OO; ^P<0.001 vs OO and OO+Indo
B
TG
Triglycerides (mg/g)
90
75
60
45
30
15
0
^
^
^
OO
FO
OO+Indo
FO+Indo
^P<0.001 vs OO
C
FC
Free cholesterol (mg/g)
4
3
2
1
0
OO
FO
OO+Indo
FO+Indo
D
FFA
Free fatty acids (mg/g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OO
FO
OO+Indo
FO+Indo

Figure 2

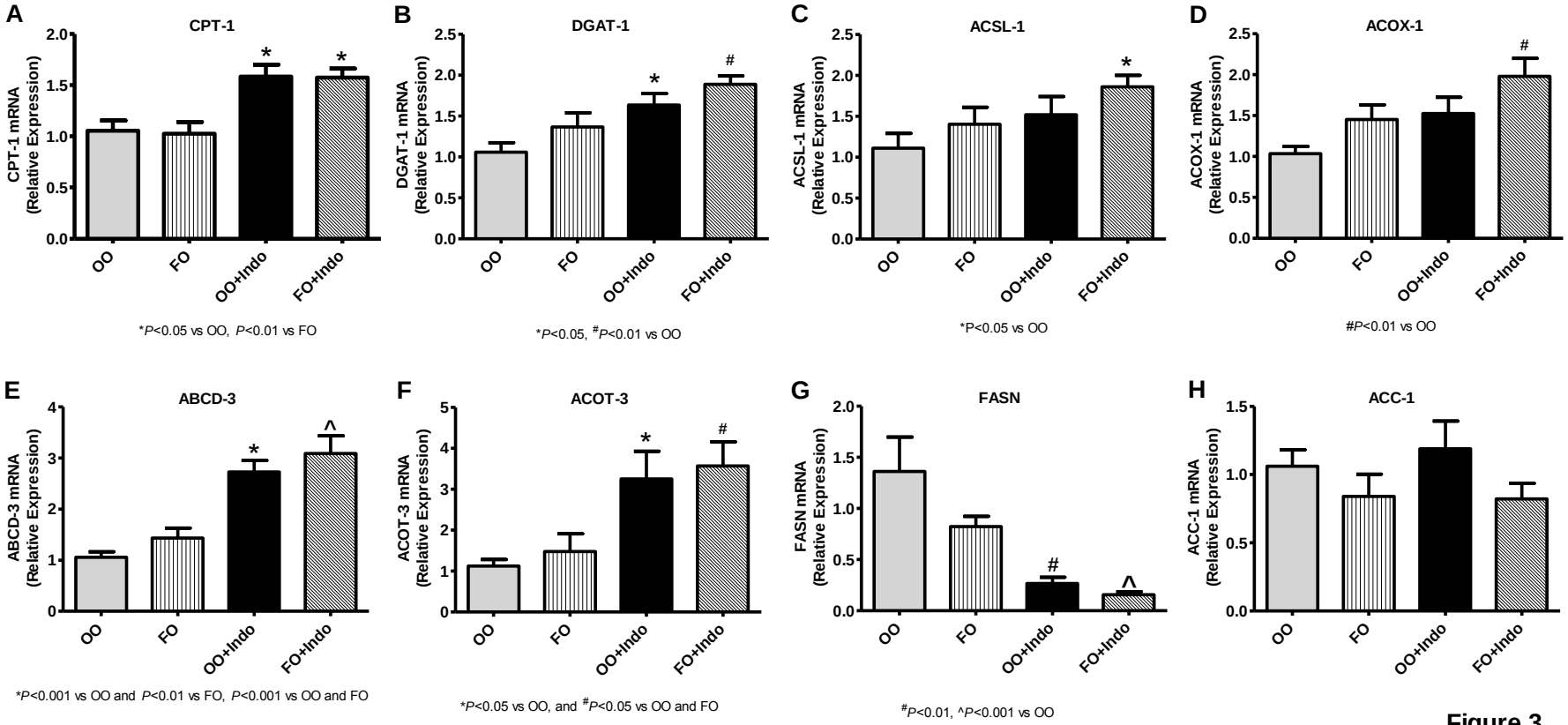

A
CPT-1
CPT-1 mRNA (Relative Expression)
2.0
1.5
1.0
0.5
0.0
*
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO, P<0.01 vs FO
B
DGAT-1
DGAT-1 mRNA (Relative Expression)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
OO
FO
OO+Indo
FO+Indo
*P<0.05, #P<0.01 vs OO
C
ACSL-1
ACSL-1 mRNA (Relative Expression)
2.5
2.0
1.5
1.0
0.5
0.0
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO
D
ACOX-1
ACOX-1 mRNA (Relative Expression)
2.5
2.0
1.5
1.0
0.5
0.0
#
OO
FO
OO+Indo
FO+Indo
#P<0.01 vs OO
E
ABCD-3
ABCD-3 mRNA (Relative Expression)
4
3
2
1
0
*
^
OO
FO
OO+Indo
FO+Indo
*P<0.001 vs OO and P<0.01 vs FO, P<0.001 vs OO and FO
F
ACOT-3
ACOT-3 mRNA (Relative Expression)
5
4
3
2
1
0
*
#
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO, and #P<0.05 vs OO and FO
G
FASN
FASN mRNA (Relative Expression)
2.0
1.5
1.0
0.5
0.0
#
^
OO
FO
OO+Indo
FO+Indo
#P<0.01, ^P<0.001 vs OO
H
ACC-1
ACC-1 mRNA (Relative Expression)
1.5
1.0
0.5
0.0
OO
FO
OO+Indo
FO+Indo

Figure 3

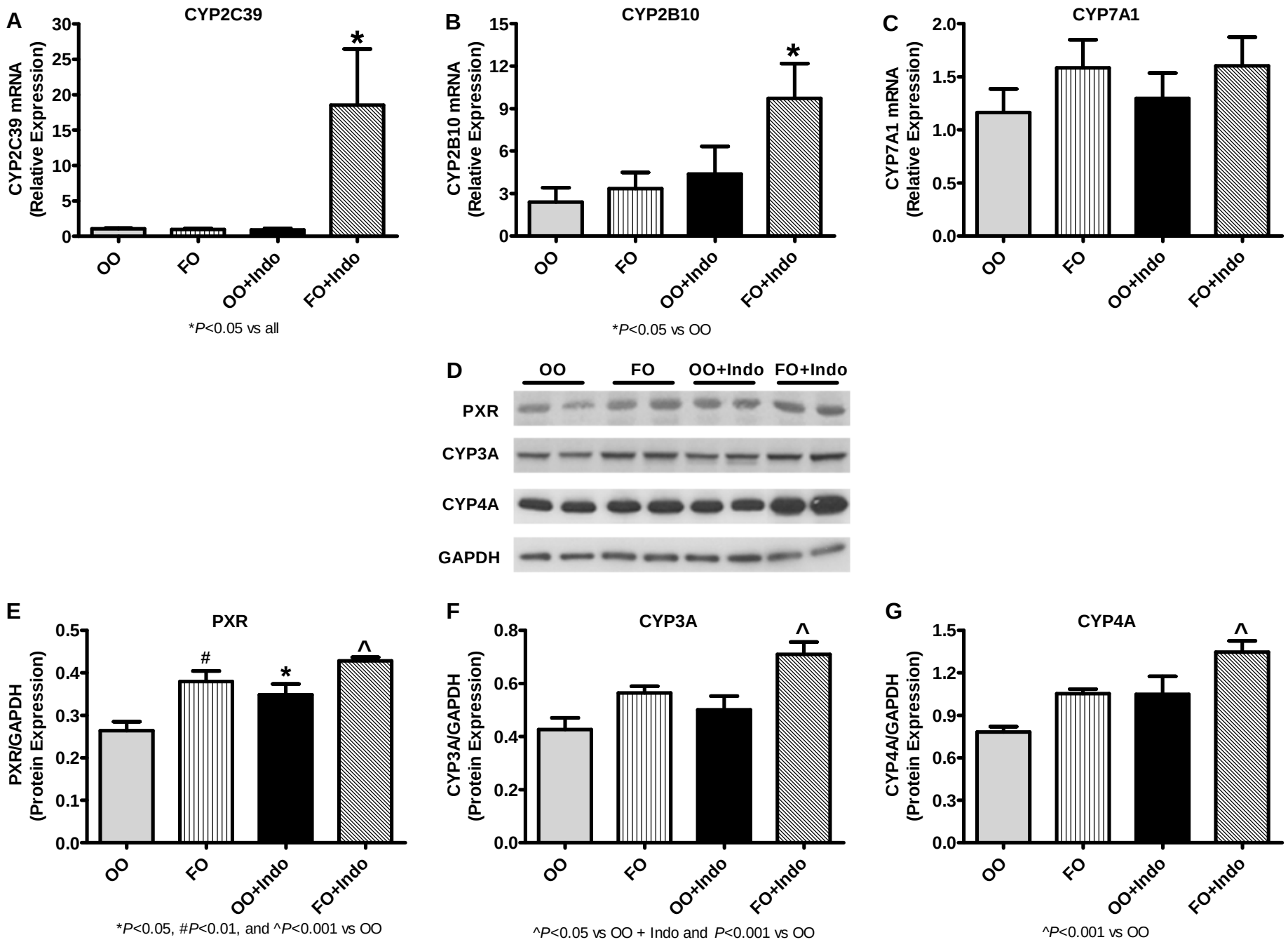

A
CYP2C39
CYP2C39 mRNA (Relative Expression)
30
25
20
15
10
5
0
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs all
B
CYP2B10
CYP2B10 mRNA (Relative Expression)
15
12
9
6
3
0
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO
C
CYP7A1
CYP7A1 mRNA (Relative Expression)
2.0
1.5
1.0
0.5
0.0
OO
FO
OO+Indo
FO+Indo
D
OO
FO
OO+Indo
FO+Indo
PXR
CYP3A
CYP4A
GAPDH
E
PXR
PXR/GAPDH (Protein Expression)
0.5
0.4
0.3
0.2
0.1
0.0
#
*
^
OO
FO
OO+Indo
FO+Indo
*P<0.05, #P<0.01, and ^P<0.001 vs OO
F
CYP3A
CYP3A/GAPDH (Protein Expression)
0.8
0.6
0.4
0.2
0.0
^
OO
FO
OO+Indo
FO+Indo
^P<0.05 vs OO + Indo and P<0.001 vs OO
G
CYP4A
CYP4A/GAPDH (Protein Expression)
1.5
1.2
0.9
0.6
0.3
0.0
^
OO
FO
OO+Indo
FO+Indo
^P<0.001 vs OO

Figure 4

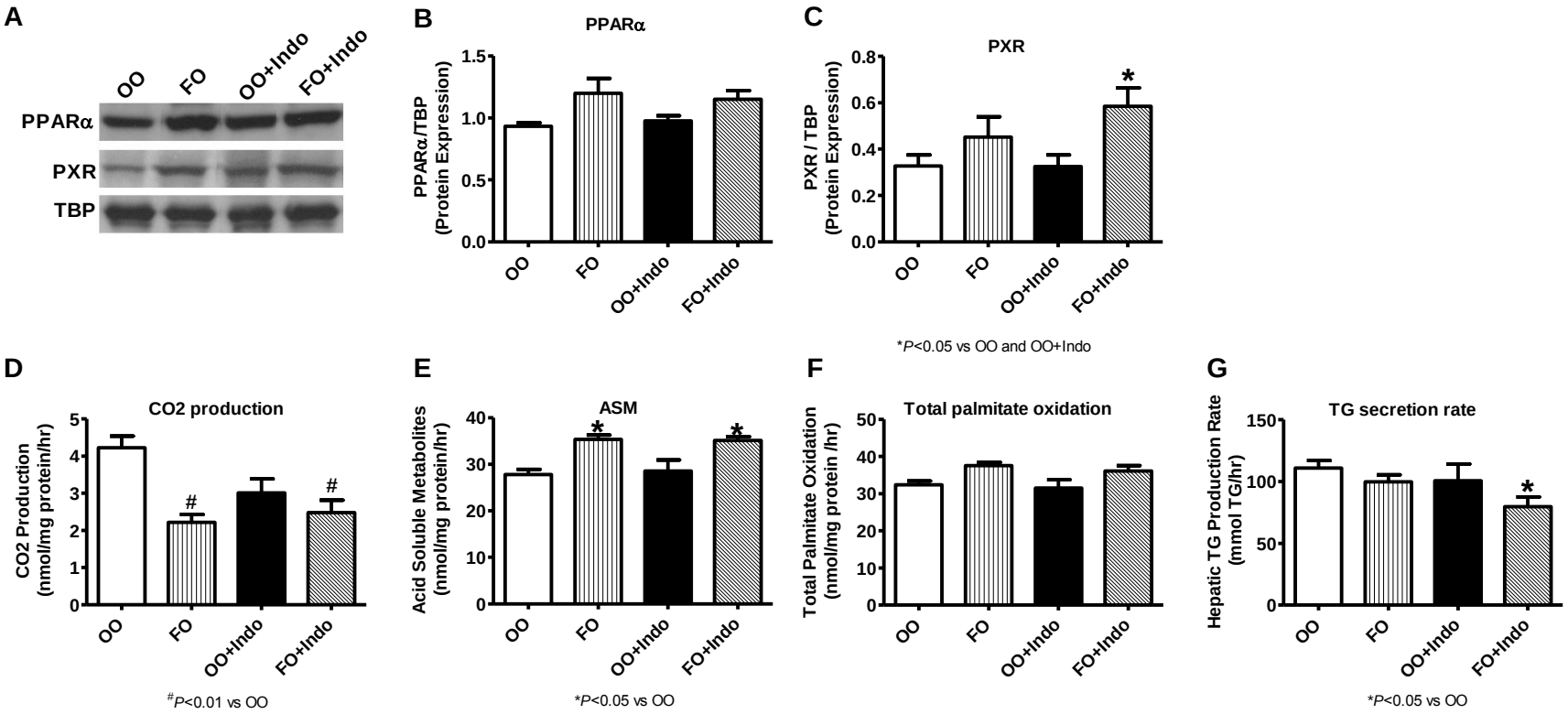

A
OO
FO
OO+Indo
FO+Indo
PPARα
PXR
TBP
B
PPARα
PPARα/TBP (Protein Expression)
1.5
1.0
0.5
0.0
OO
FO
OO+Indo
FO+Indo
C
PXR
PXR / TBP (Protein Expression)
0.8
0.6
0.4
0.2
0.0
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO and OO+Indo
D
CO2 production
CO2 Production (nmol/mg protein/hr)
5
4
3
2
1
0
#
#
OO
FO
OO+Indo
FO+Indo
#P<0.01 vs OO
E
ASM
Acid Soluble Metabolites (nmol/mg protein/hr)
40
30
20
10
0
*
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO
F
Total palmitate oxidation
Total Palmitate Oxidation (nmol/mg protein /hr)
50
40
30
20
10
0
OO
FO
OO+Indo
FO+Indo
G
TG secretion rate
Hepatic TG Production Rate (mmol TG/hr)
150
100
50
0
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO

Figure 5

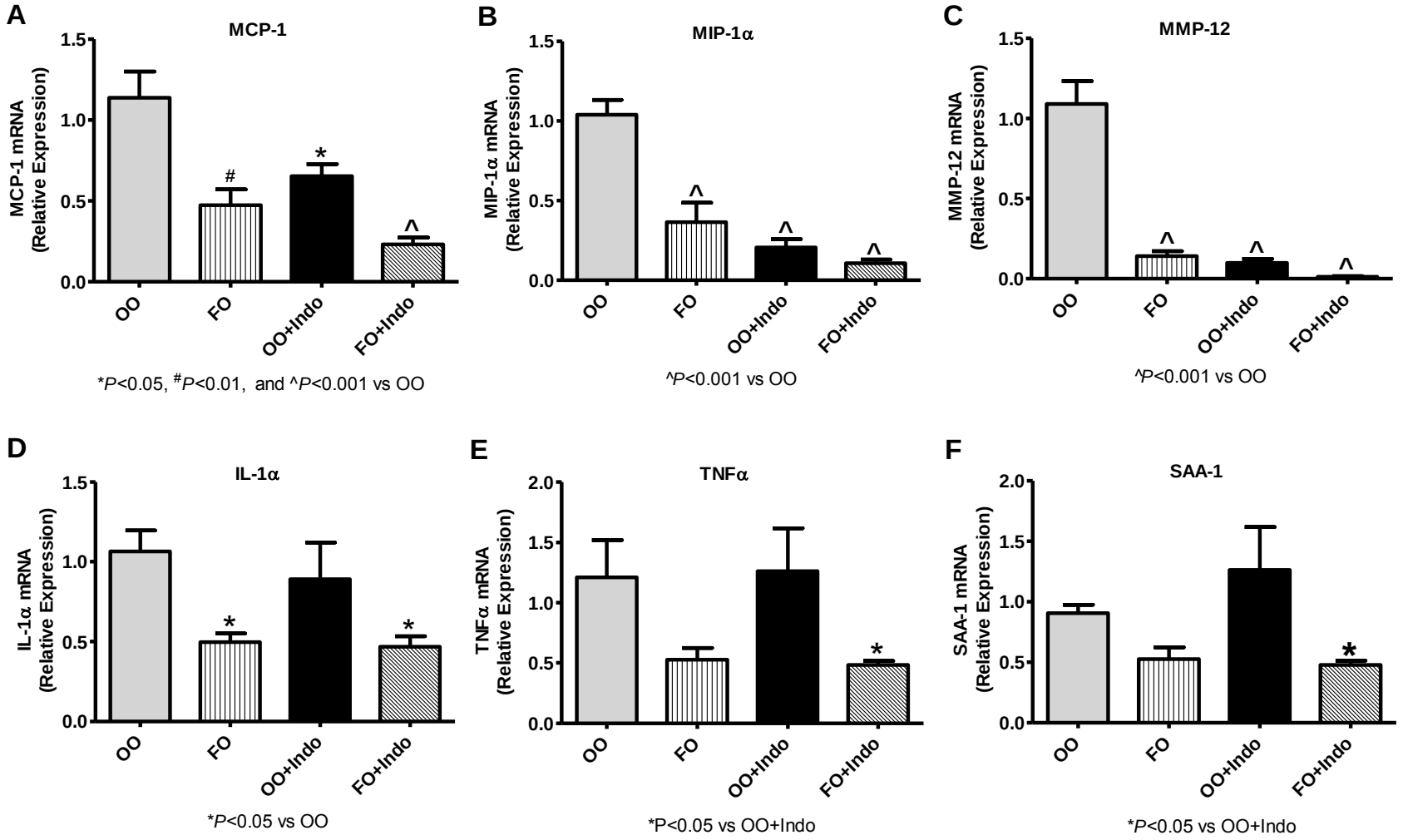
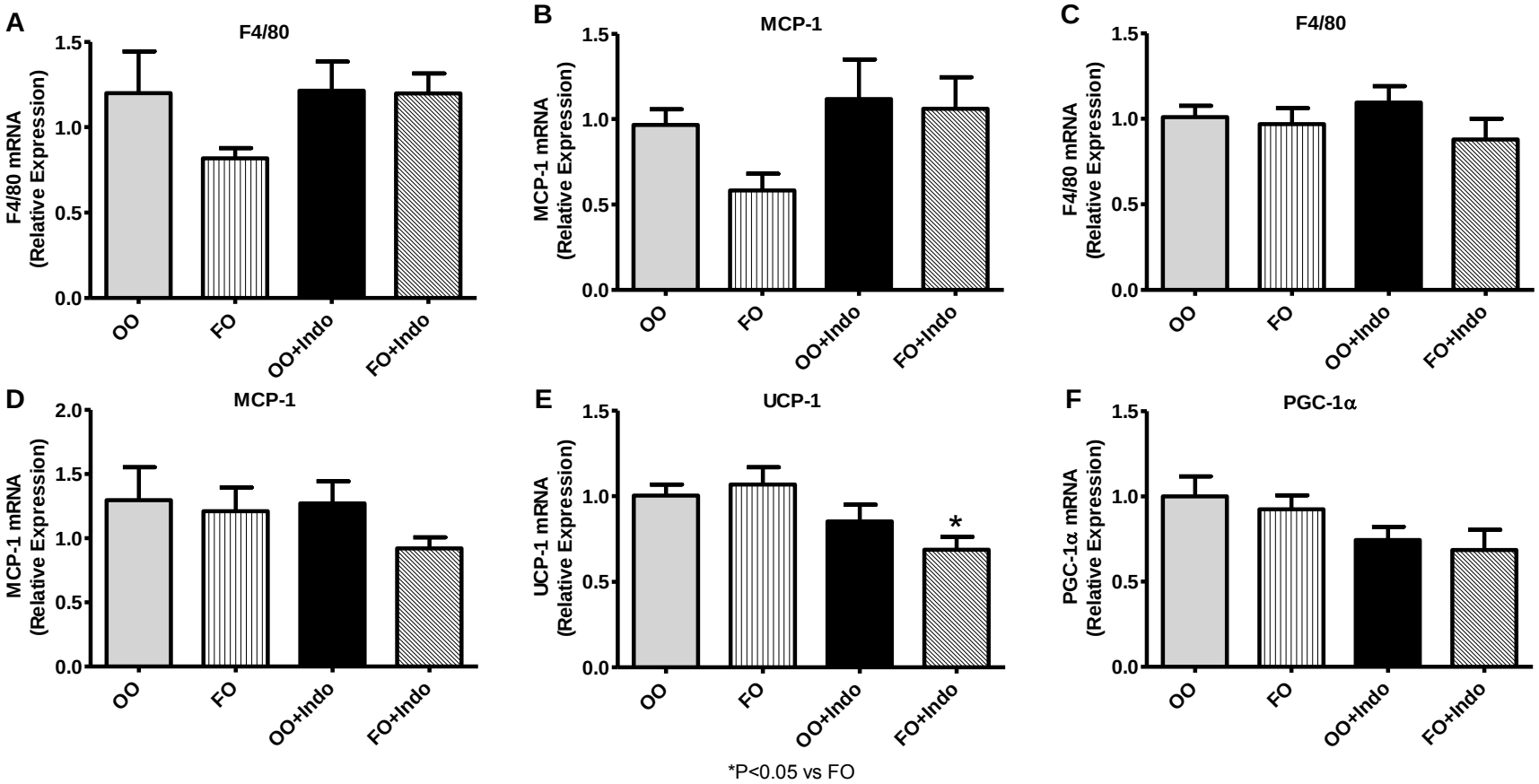

A
MCP-1
MCP-1 mRNA
(Relative Expression)
1.5
1.0
0.5
0.0
#
*
^
OO
FO
OO+Indo
FO+Indo
*P<0.05, #P<0.01, and ^P<0.001 vs OO
B
MIP-1α
MIP-1α mRNA
(Relative Expression)
1.5
1.0
0.5
0.0
^
^
^
OO
FO
OO+Indo
FO+Indo
^P<0.001 vs OO
C
MMP-12
MMP-12 mRNA
(Relative Expression)
1.5
1.0
0.5
0.0
^
^
^
OO
FO
OO+Indo
FO+Indo
^P<0.001 vs OO
D
IL-1α
IL-1α mRNA
(Relative Expression)
1.5
1.0
0.5
0.0
*
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO
E
TNFα
TNFα mRNA
(Relative Expression)
2.0
1.5
1.0
0.5
0.0
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO+Indo
F
SAA-1
SAA-1 mRNA
(Relative Expression)
2.0
1.5
1.0
0.5
0.0
*
OO
FO
OO+Indo
FO+Indo
*P<0.05 vs OO+Indo

Figure 6



*P<0.05 vs FO

Figure 7

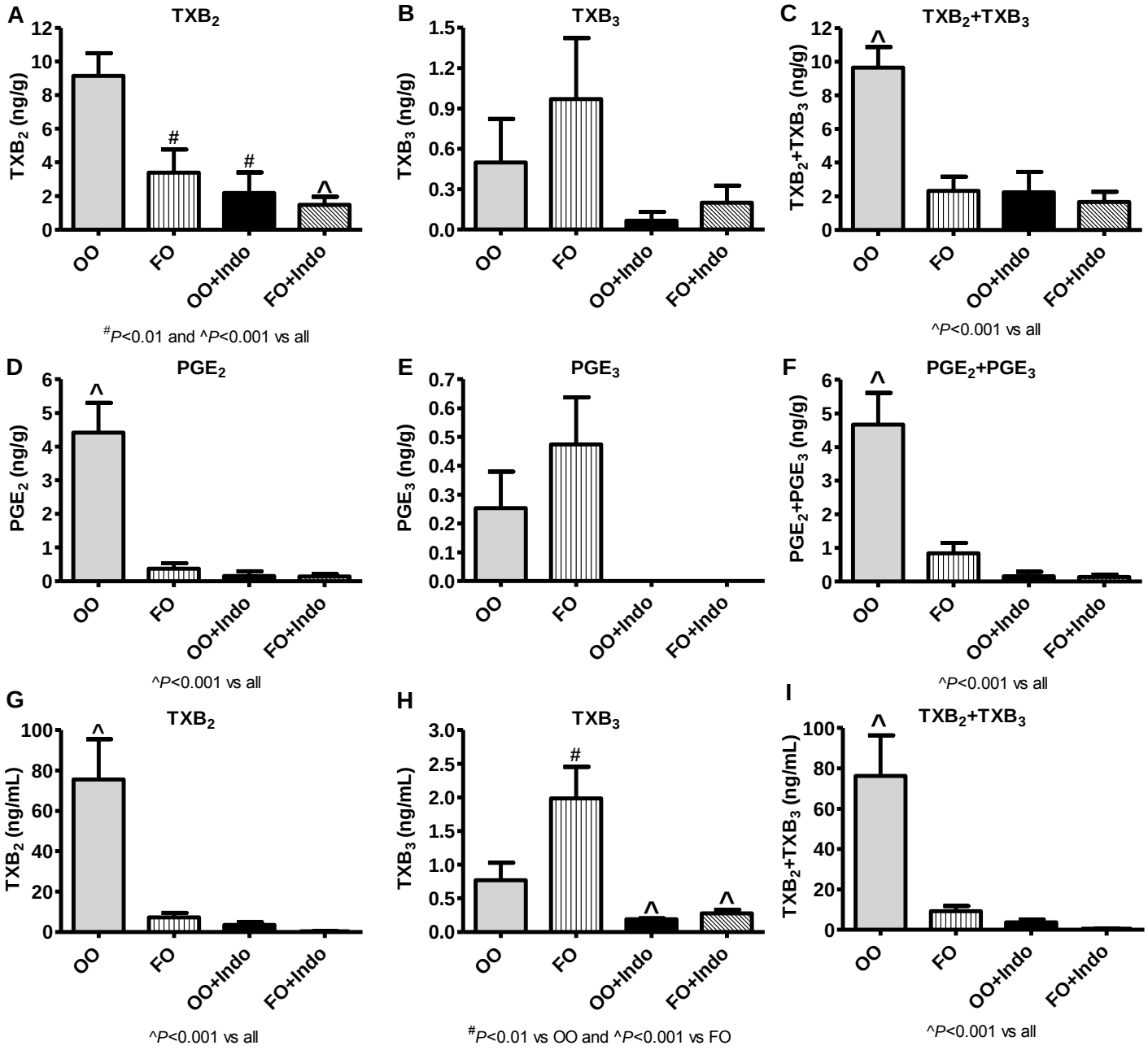

A
TXB2
TXB2 (ng/g)
OO
FO
OO+Indo
FO+Indo
#P<0.01 and ^P<0.001 vs all
B
TXB3
TXB3 (ng/g)
C
TXB2+TXB3
TXB2+TXB3 (ng/g)
^P<0.001 vs all
D
PGE2
PGE2 (ng/g)
^P<0.001 vs all
E
PGE3
PGE3 (ng/g)
F
PGE2+PGE3
PGE2+PGE3 (ng/g)
^P<0.001 vs all
G
TXB2
TXB2 (ng/mL)
^P<0.001 vs all
H
TXB3
TXB3 (ng/mL)
#P<0.01 vs OO and ^P<0.001 vs FO
I
TXB2+TXB3
TXB2+TXB3 (ng/mL)
^P<0.001 vs all

Figure 8