

Role of PPAR α in the attenuation of bile acid-induced apoptosis by omega-3 long-chain polyunsaturated fatty acids in cultured hepatocytes

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BACKGROUND: Omega-3 long-chain polyunsaturated fatty acids (ω 3PUFA) have been shown to be antiinflammatory in the attenuation of hepatocellular injury. Peroxisome proliferator-activated receptor alpha (PPAR α) is a nuclear receptor transcription factor that inhibits the activation of nuclear factor κ B, thereby repressing inflammation, and ω PUFA are PPAR α ligands. The purpose of this study was to determine if ω 3PUFA attenuate bile acid-induced apoptosis via PPAR α .

METHODS: Human hepatocellular carcinoma (HepG2) cells were treated with chenodeoxycholic acid (CDCA) \pm ω 3PUFA. Activation of PPAR α was evaluated, and expression of PPAR α , farnesoid X receptor, liver X receptor alpha (LXR α), and retinoid X receptor mRNA was evaluated by reverse-transcriptase PCR.

RESULTS: PPAR α activation was increased in HepG2 cells treated with ω 3PUFA, and decreased in the presence of CDCA when compared with untreated cells. PPAR α mRNA was reduced by 67% with CDCA and restored to the level of control with ω 3PUFA. LXR α mRNA increased twofold with CDCA treatment and was significantly reduced by ω 3PUFA.

CONCLUSION: Expression of PPAR α , as well as LXR α mRNA levels, was reduced with CDCA treatment and restored with the addition of ω 3PUFA. These results suggest that PPAR α and LXR α may be mediators by which ω 3PUFA attenuate bile acid-induced hepatocellular injury.

Peroxisome proliferator-activated receptor alpha (PPAR α) is a nuclear receptor transcription factor that regulates lipid metabolism. When bound to a ligand, PPAR α forms a heterodimer with retinoid X receptor (RXR) that binds to the peroxisome proliferator response element in the promoter region of target genes and modulates transcription as depicted in **Figure 1**. Activation of PPAR α inhibits nuclear factor κ B (NF κ B) activation, and consequently repressing inflammation (1,2). Hepatic nuclear receptor RXR has also been shown to be important in hepatic responses to inflammation (3). Alternatively, NF κ B activation increases expression of antiapoptotic genes, such as the caspase-8 inhibitor c-FLIP, which

could lead to resistance to Fas ligand and/or tumor necrosis factor-dependent apoptosis (4). Previous studies have shown omega-3 long-chain polyunsaturated fatty acids (ω 3PUFA) to be a PPAR α ligand, thereby inhibiting NF κ B activation (2). In studies where PPAR α null mouse hepatocytes treated with nafenopin (a peroxisome proliferator), apoptosis was not suppressed, although treatment with tumor necrosis factor- α was able to suppress apoptosis in PPAR α null hepatocytes (5). These studies demonstrated that PPAR α mediates hepatocyte apoptosis, yet high concentrations of tumor necrosis factor- α can modulate and/or negate this response (5). These results suggest that PPAR α inhibition of NF κ B may play an important role in the overall cellular innate immune response and cell survival. The pathways affected by PPAR α and NF κ B are complex and in some cases, seemingly contradictory. These contradictory effects of NF κ B on apoptosis have perplexed investigators studying interferon-mediated activation of NF κ B, where this results in antiviral and proapoptotic activity. There is also evidence that micro-RNAs may have an important role in this complex signaling pathway (6). Finally, PPAR α is known to inhibit apoptosis via NF κ B-independent pathways, such as upregulation of the 14-3-3 proteins (7). The suppression of proinflammatory cytokine transcription by PPAR α via NF κ B would support our hypothesis, but the NF κ B-mediated suppression of antiapoptotic pathways would suggest that ω 3PUFA activation of PPAR α could result in increased apoptosis.

Omega-3 PUFA have been shown to be antiinflammatory and immunomodulatory in the attenuation of cellular injury (8–13). Recently, there has been much interest in the use of fish oil contacting ω 3PUFA to treat parenteral nutrition-associated liver disease (PNALD) in children (14). Interestingly, ω 3PUFA have been shown to paradoxically inhibit apoptosis and sensitize the cells to apoptosis (15–18). It is established that the composition and levels of free fatty acids can significantly alter cell survival pathways. Free fatty acids have been shown to inhibit serum-starved apoptosis in a murine cell model via the G-coupled protein receptor, GPR120 (19). In these experiments, both saturated and unsaturated free fatty acid-enhanced

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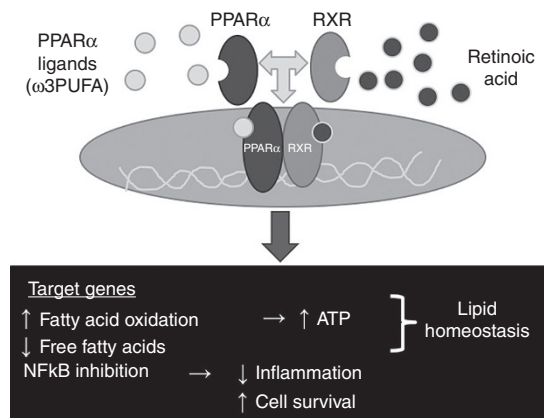


Figure 1. PPAR α transcriptional activity. This figure is an illustration of PPAR α transcription and its effects on lipid homeostasis and NF κ B activation. PPAR α , peroxisome proliferator-activated receptor alpha.

cell survival, but linolenic acid, palmitoleic acid, and docosahexaenoic acid (DHA) had the most potent effects on cell survival (20). In addition, GPR120 has recently been shown to be a receptor or sensor for ω 3PUFA, mediating regulation of inflammation, and inhibiting both toll-like receptor 2/3/4 and tumor necrosis factor- α cascades (21). Both NF κ B and c-Jun N-terminal kinases have been shown to play a role in the upregulation of death receptors in the presence of noxious stimuli (22,23).

In the present studies, we hypothesized that ω 3PUFA would modulate PPAR α activation and/or mRNA levels resulting in the attenuation of hydrophobic bile acid-induced apoptosis via PPAR α downstream effects. Expression of liver X receptor alpha (LXR α) and farnesoid X receptor (FXR) was also studied, as these nuclear receptors are recognized mediators of diverse metabolic pathways in liver, including inflammation, apoptosis and fibrosis (24,25).

RESULTS

PPAR α Activity

As shown in **Figure 2**, treatment of Human hepatocellular carcinoma (HepG2) cells with ω 3PUFA alone resulted in increased PPAR α activity compared with controls and levels were statistically equal to that observed with positive control (recombinant PPAR α). Treatment with ω 3PUFA was increased 37% over untreated cells and DNA binding of PPAR α was decreased by 45% in the presence of chenodeoxycholic acid (CDCA) ($P < 0.001$). There was a modest trend to restoration (only decreased by 30% as compared with 45%) of PPAR α activity to untreated control levels with the addition of ω 3PUFA in the setting of CDCA treatment.

PPAR α , LXR α , FXR, and RXR mRNA Levels

Treatment with CDCA alone resulted in 67% reduction in PPAR α mRNA, as compared with cells treated with a vehicle control (**Figure 3a**). Treatment with CDCA alone resulted in a twofold increase in LXR α mRNA that was attenuated by the addition of ω 3PUFA (**Figure 3b**). No significant changes

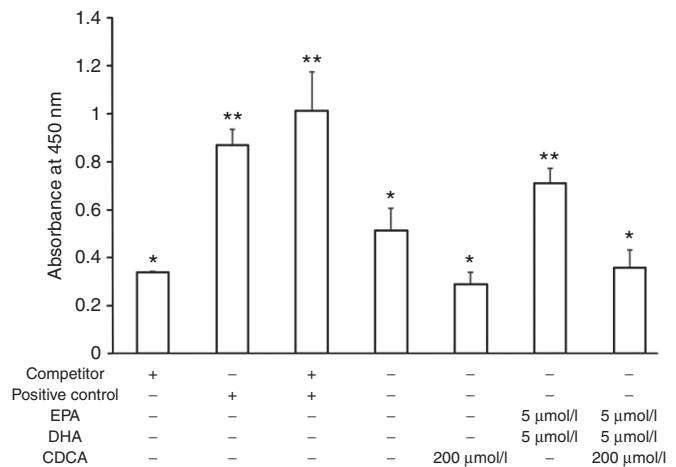


Figure 2. PPAR α activity. PPAR α activity in HepG2 cells treated with CDCA with and without ω 3PUFA. Values are based on a fold change relative to the vehicle control. Statistical significance was determined using an ANOVA with Tukey's LSD. Each bar represents mean \pm SEM for data from three culture wells. Statistical significance was determined using an ANOVA with Tukey's LSD. Means of data from various treatment conditions with different letters above the bars are significantly different from each other at $P < 0.05$ by ANOVA. Those bars sharing the same symbol or no symbol are not significantly different from each other. PPAR α , peroxisome proliferator-activated receptor alpha; CDCA, chenodeoxycholic acid; ω 3PUFA, omega-3 long-chain polyunsaturated fatty acids. LSD, least significant difference.

in RXR or FXR mRNA levels were observed with either treatment (data not shown).

Role of PPAR α in the Attenuation of Bile Acid-Induced Apoptosis

As shown in **Figure 4**, HepG2 cells treated with ω 3PUFA, in addition to the CDCA challenge, exhibited a 47% attenuation of apoptosis, as reflected by caspase-3/7, when compared with cells treated with CDCA alone. This ω 3PUFA protective effect was not observed with blockade of PPAR α with specific antibody. With antibody dose escalation, there was no significant difference in caspase-3/7 with increasing doses of antibody. These results suggest that the attenuation of apoptosis occurs via PPAR α -dependent pathways.

DISCUSSION

The present studies support a role for PPAR α in protection from bile acid-induced apoptosis in hepatocytes and may, at least partially, mediate the attenuation of apoptosis by ω 3PUFA, which may represent one mechanism for the positive effect of ω 3PUFA treatment in PNALD. Activation and mRNA levels of PPAR α were reduced by treatment with the lipophilic bile acid, CDCA, at a dose associated with hepatocyte apoptosis. When hepatocytes were exposed to ω 3PUFA alone, PPAR α activity and mRNA levels were increased. When cells were treated with CDCA with the addition of ω 3PUFA, PPAR α mRNA levels were similar to those of control cells, and there was a trend to restoration of PPAR α activation. Our findings are consistent with studies of CFTR-knockout mice and isolated macrophages from CFTR-knockout mice in which pretreatment with DHA resulted in increased PPAR α mRNA levels. This increase was blocked by the addition of

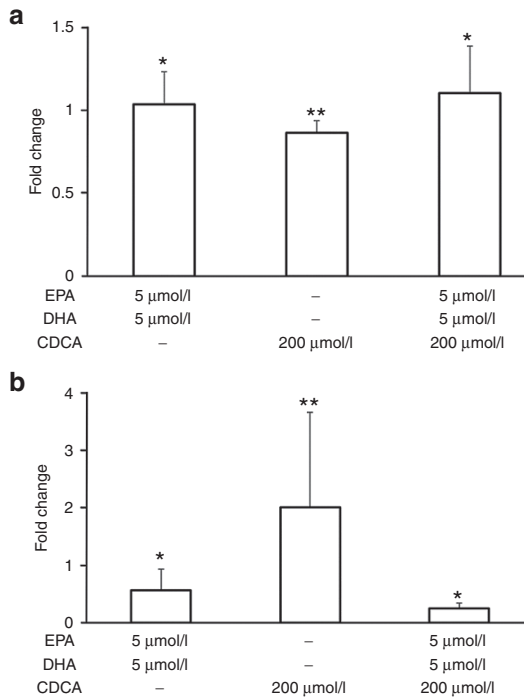


Figure 3. PPARα and LXRα mRNA levels. (a) PPARα, (b) LXRα mRNA levels were measured by quantitative RT-PCR. Values are based on a fold change relative to the vehicle control. Statistical significance was determined using an ANOVA with Tukey's LSD. Each bar represents mean ± SEM for data from three culture wells. Statistical significance was determined using an ANOVA with Tukey's LSD. Means of data from various treatment conditions with different letters above the bars are significantly different from each other at *P* < 0.05 by ANOVA. Those bars sharing the same symbol or no symbol are not significantly different from each other. PPARα, peroxisome proliferator-activated receptor alpha; LXRα, liver X receptor alpha; RT-PCR, reverse-transcriptase PCR. LSD, least significant difference.

a PPARα antagonist (26,27). In contrast, studies in a mouse model of PNALD using PPARα-knockout and wild-type mice resulted in increased hepatic triglyceride concentrations in both WT and KO mice treated with PN with the addition of ω3PUFA and ω6PUFA as compared with chow fed mice (28). However, animals receiving ω3PUFA had lower hepatic triglyceride content compared with animals fed ω6PUFA in both WT and KO mice. Therefore, the investigators concluded that ω3PUFA attenuation of hepatic triglyceride accumulation in this model of PNALD is not via PPARα-dependent pathways (28). While the effects of ω3PUFA on hepatic triglyceride accumulation may not be PPARα dependent in this *in vivo* model of PNALD, we have shown in the present studies in an *in vitro* model that bile acid-induced apoptosis often associated with PNALD is attenuated by ω3PUFA via PPARα-dependent pathways. This is not surprising in light of the complex and multifactorial nature of PNALD pathogenesis, which involves hepatocellular injury, inflammation, and apoptosis in addition to steatosis. Our data support the hypothesis that ω3PUFA are functioning, at least partially, via PPARα-dependent mechanisms to attenuate direct bile acid-induced apoptosis. The mechanisms by which ω3PUFA

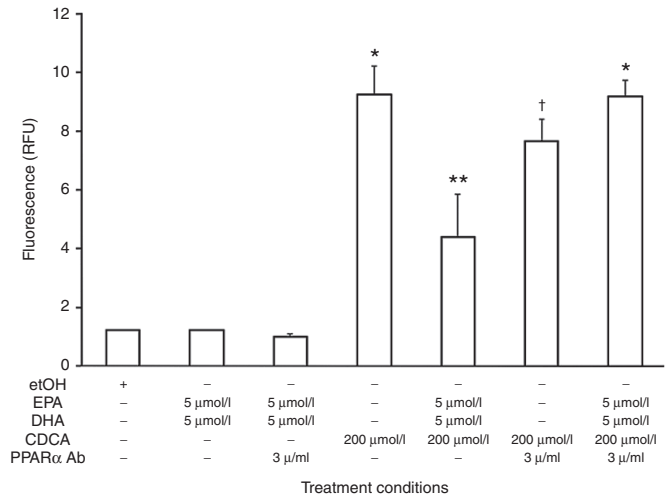


Figure 4. Caspase-3/7 activity. Bile acid-induced apoptosis evaluated by caspase-3/7 with and without the addition of an antibody specific to PPARα with ω3PUFA treatment. Data are represented based on relative fluorescence units above control. Each bar represents mean ± SEM for data from three independent experiments. Statistical significance was determined using an ANOVA with Tukey's LSD. Means of data from various treatment conditions with different letters above the bars are significantly different from each other at *P* < 0.05 by ANOVA. Those bars sharing the same symbol or no symbol are not significantly different from each other. PPARα, peroxisome proliferator-activated receptor alpha; ω3PUFA, omega-3 long-chain polyunsaturated fatty acids. LSD, least significant difference.

reduce hepatic steatosis may indeed occur independently of PPARα.

For these experiments, we chose to use an equal molar ratio of eicosapentaenoic acid to DHA, because our group has previously shown eicosapentaenoic acid and DHA to be synergistic in the attenuation of bile acid induce apoptosis (29). We observed a 67% reduction in PPARα mRNA levels when cells were treated with CDCA as compared with cells treated with a vehicle control. The addition of ω3PUFA resulted in restoration to the level of control cells. We did not observe a significant difference in RXR mRNA levels, which was not unexpected, since abundance of RXR and its ligand, *cis* retinoic acid, typically precludes it from becoming rate limiting in its role as a heterodimer partner with transcription factors.

Treatment with CDCA alone resulted in a twofold increase in LXRα mRNA levels that was attenuated by the addition of ω3PUFA. It is noteworthy that LXRα has been shown to have specific roles in regulating the innate immune response (30). Also, studies in LXRα null macrophages have shown that LXRα has an important role in both innate and adaptive immunity, specifically in apoptosis (31). Cancer cells of diverse origin may be programmed to enter apoptosis by LXRα activation (32). Therefore, it is possible that LXRα may be an important mediator of CDCA-induced apoptosis, and its suppression by ω3PUFA may represent an additional pathway for the beneficial effect of ω3PUFA on apoptosis. Interestingly, FXR mRNA abundance was not affected by CDCA or ω3PUFA treatment.

In summary, these studies suggest roles for PPARα, as well as LXRα, in the regulation of bile acid-induced apoptosis in hepatocytes and its suppression by ω3PUFA. It is possible that

PPAR α activation and LXR α suppression may be promising mechanisms by which ω 3PUFA attenuate the bile acid-induced hepatocellular injury that occurs in cholestatic liver disease, such as that seen in PNALD. However, these conclusions must be tempered by several caveats. First, these studies were performed in a cell culture system using hepatoma-derived hepatocytes. Second, we explored but one mechanism of cell injury, bile acid toxicity, and its induction of apoptosis, whereas multiple pathogenic mechanisms are operative in clinical PNALD. Finally, more work will be required to further define additional potential pathways involved in these observed ω 3PUFA effects and to translate these findings to useful therapy for PNALD in human infants.

METHODS

Cell Culture

Because this was an *in vitro* study and did not involve the use of human subjects or animals, the University of Tennessee Health Science Center did not require approval by the Institutional Review Board. Cell culture experiments were performed using HepG2 cells obtained from the American Type Culture Collection (Rockville, MD). Cell culture experiments used standard medium, incubation conditions, and passages according to the supplier's instructions. HepG2 cells were treated with CDCA (200 μ mol/l) (Sigma-Aldrich, St. Louis, MO) with and without the addition of ω 3PUFA (5 μ mol/l eicosapentaenoic acid combined with 5 μ mol/l DHA) (Sigma-Aldrich). Concentrations of CDCA and ω 3PUFA for these experiments were based on our previously published work (29).

PPAR α Activation

Activation of PPAR α was evaluated by isolation of nuclear protein after 4-h treatment and measured by assessing transcription factor activity in a nonradioactive, sensitive ELISA method for detecting PPAR α DNA binding activity in nuclear extracts (PPAR α Transcription Factor Assay Kit, Cayman Chemical, Ann Arbor, MI). This kit contained a positive control of clarified cell lysate and a competitor of double-strand DNA.

PPAR α mRNA Analysis

HepG2 cells were treated with 200 μ mol/l CDCA \pm ω 3PUFA for 0.5 h, and PPAR α , LXR α , FXR, and RXR mRNA levels were evaluated by reverse-transcriptase PCR using an ABI 7500 Sequence Detector and SYBR Green PCR Master Mix according to the manufacturer's instructions. Levels of mRNA were normalized to 18s ribosomal RNA quantified simultaneously in a multiplex reverse-transcriptase PCR reaction. Specific primers were designed for the genes of interest (PPAR α , FXR, LXR α , and RXR). See [Table 1](#) for primer sequences. All samples were analyzed in triplicates.

Table 1. Reverse-transcriptase PCR (RT-PCR) primer sequences

Primer	Sequence
18s	F: 5'-CTCAACACGGGAAACCTCAC-3' R: 5'-TTATCGGAATTAACCAGACAAATCG-3'
PPAR α	F: 5'-GTAGAATCTGCGGGACAAG-3' R: 5'-TGTGACATCCCGACAGAAAG-3'
FXR	F: 5'-CTACCAGGATTCAGACTTTGGAC-3' R: 5'-GAACATAGCTTCAACCCGACAGAC-3'
LXR α	F: 5'-CGCTCGAGATGCTCTGTGGCTGG-3' R: 5'-CGCTCTAGATCATTCGTGCACATCC-3'
RXR	F: 5'-AAGGACCGAACGAGAATGA-3' R: 5'-ATCCTCTCCACGGCATGT-3'

Caspase 3/7 Activity

In order to determine if the attenuation of bile acid-induced apoptosis by ω 3PUFA occurs via PPAR α -dependent pathways, HepG2 cells were treated with a PPAR α polyclonal antibody (PPAR α H-98: sc-9000) purchased from Santa Cruz Biotechnology (Dallas, TX), and apoptosis was evaluated using caspase-3/7. Cultured HepG2 cells were treated with ω 3PUFA (eicosapentaenoic acid 5 μ mol/l + DHA 5 μ mol/l) alone; 200 μ mol/l CDCA alone; and then with the combinations ω 3PUFA, and varying concentrations of PPAR α antibody (3–10 μ g/ml). Apoptosis was evaluated using the Apo-ONE Homogeneous Caspase-3/7 Assay purchased from Promega Corporation (Madison, WI) and performed according to the manufacturer's instructions. HepG2 cells were treated for 12 h followed by the addition of caspase-3/7 reagent and incubation for 4 h in the dark on a rocking shaker at low speed. Results were read at fluorescein 485/535nm with a Victor 2, Perkin-Elmer Wallace 1420 multilabel counter (Shelton, CT) as previously described (29).

Statistical Analysis

All data represent at least three separate and independent experiments. Data are depicted as mean \pm SEM. A one-way ANOVA was used to compare differences between groups and a *post-hoc* Tukey's HSD (honestly significant difference) test was used to correct for multiple comparisons. A two-tailed *P* value of 0.05 was used to reject the null hypothesis.

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