Potential Relationship between Hepatobiliary Osteopontin and Peroxisome Proliferator–Activated Receptor α Expression following Ethanol-Associated Hepatic Injury In Vivo and In Vitro

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Osteopontin (OPN) up-regulation is known to mediate hepatic inflammation in a rodent model of alcoholic liver disease (ALD) and alcohol ingestion is reported to inhibit hepatic peroxisome proliferator–activated receptor-α (PPAR-α) activity leading to hepatic steatosis and inflammation. Therefore, the objective of this study was to investigate the potential relationship between the anti-inflammatory PPAR-α and proinflammatory OPN in rats and mice livers, and cell cultures of hepatocytes and biliary epithelium. Experiments were designed to evaluate the influence of ethanol (EtOH), lipopolysaccharide (LPS), and acetaldehyde (ACA) on OPN and PPAR-α expression levels in vivo (rats and mice) and in vitro (hepatocytes and biliary epithelium). Adult Sprague-Dawley rats and C57BL6 mice were fed EtOH-containing Lieber-DeCarli liquid diet for 6 weeks and injected with a single dose of LPS. A combination of EtOH and LPS treated rats and mice showed significant induction of hepatic OPN expression compared with the controls. Similarly, cells exposed to physiologic doses of EtOH, LPS, a combination of EtOH and LPS, and ACA resulted in increased OPN protein and mRNA expression. Rats and mice in ALD model and cells treated with EtOH and ACA showed downregulation of PPAR-α mRNA. Also, DNA binding activity of PPAR-α to PPAR response element was significantly reduced following treatment. Overexpression of PPAR-α rescued the reduced PPAR-α activity and PPAR-α agonist, bezafibrate, elevated PPAR-α activity after treatment of EtOH, LPS, and ACA when cells were exposed by bezafibrate. To further delineate the potential relationship between OPN and PPAR-α, OPN−/− mice showed no change of PPAR-α mRNA level although wild-type mice showed downregulation of PPAR-α mRNA after EtOH treatment. In conclusion, the current study suggests that OPN is induced by EtOH and its metabolite ACA and opposite relationship likely exist between PPAR-α and OPN expression within the liver during ALD.

Key Words: bezafibrate; ethanol; osteopontin; peroxisome proliferator–activated receptor-α.

Osteopontin (OPN) is a phosphorylated glycoprotein associated with pathologic events such as inflammation (Apte et al., 2005; Banerjee et al., 2006a, b; Diao et al., 2004; Ramaiah and Jaeschke, 2007; Ramaiah and Rittling, 2007, 2008; Sahai et al., 2004; Scatena et al., 2007), cancer progression (Chakraborty et al., 2006; El-Tanani et al., 2006), metastasis (Denhardt et al., 2001, 2003; Nemoto et al., 2004; Ramaiah and Rittling, 2007), cardiovascular diseases (Majumdar et al., 2007), obesity (Gómez-Ambrosi et al., 2007), and osteogenesis (Amir et al., 2007; Denhardt and Noda, 1998; Qin et al., 2004). OPN is known to be secreted from immune cells, epithelial tissue, smooth muscle cells, osteoblasts, and tumor cells suggesting its potential role in these and other locations (Ramaiah and Rittling, 2007, 2008; Sodek et al., 2000). The ability of OPN to function is based on its sequences that bind to receptors including integrins and CD44 (Yokosaki et al., 1999, 2005). OPN can be modified by the different splicings and/or post-translational modifications such as glycosylation, phosphorylation, and transglutamination (Christensen et al., 2007; Kaartinen et al., 1997; Sørensen and Petersen, 1995). There are also cleavage sites in OPN mediated by thrombin and matrix metallopeptases 3 and 7 (Agnihotri et al., 2001; Ramaiah and Rittling, 2007; Yokosaki et al., 1999). These various modifications of OPN protein are reported to alter the physiologic and pathologic functions of OPN (Ramaiah and Rittling, 2007).

Recent reports from our laboratory showed in vivo hepatobiliary OPN induction and implicated the role of OPN in mediating hepatic inflammation during alcoholic liver disease (ALD) (Banerjee et al., 2006a, b). Based on this, OPN induction by treatment of a variety of hepatotoxic agents associated with ALD including ethanol (EtOH), lipopolysaccharide (LPS), and acetaldehyde (ACA) was investigated in vitro as well as in vivo. EtOH, LPS, and ACA are important contributors of alcohol-induced liver pathology. ACA is the major metabolite resulting from EtOH oxidation by alcohol dehydrogenase and serves as an important contributor of ALD (Lieber, 2000; Mello et al., 2008). LPS is a well-known contributor of EtOH-mediated liver injury and the elevated level of LPS is reported in chronic...
alcoholics (Frank et al., 2004; Uesugi et al., 2002; Yamashina et al., 2005). Based on these, a systematic evaluation of OPN expression following treatment with hepatotoxic agents was investigated in this study to confirm OPN induction.

Because higher OPN expression appears to be central to the pathogenesis of hepatic inflammation during alcohol ingestion, the possible relationship of OPN with hepatic peroxisome proliferator–activated receptor-α (PPAR-α) was also tested in this study. In addition to a variety of mechanisms causing steatosis and inflammation by alcohol ingestion, the inactivation of PPAR-α is implicated (You and Crabb, 2004). PPAR-α is one of nuclear receptor proteins which interact with the retinoid X receptor (RXR) to function as a transcription factor to induce the expression of a battery of genes involved in fatty acid transport and oxidation (Crabb et al., 2004; Donohue, 2007). Alcohol consumption causes a downregulation of lipid oxidation by the inactivation of PPAR-α that regulates genes responsible for lipid metabolism leading to alcoholic fatty liver (Costet et al., 1998; Donohue, 2007). Also, the activating ability of PPAR-α ligand WY14643 was reduced by EtOH in hepatocytes (Galli et al., 2001). Because there appears to be an opposite relationship between hepatic OPN (upregulation) and PPAR-α (downregulation) expression in ALD, such a relationship was tested in both in vivo and in vitro models of ALD.

**MATERIALS AND METHODS**

**Chemicals.** Unless otherwise mentioned, all chemicals and reagents employed for all experiments described in this study were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents for western blotting and protein assay reagent for determination of total protein concentration were obtained from Bio-Rad laboratories (Hercules, CA). Reagents for real-time reverse transcription–polymerase chain reaction (RT-PCR) were purchased from Applied Biosystems (Foster City, CA). Luciferase assay reagents were obtained from Promega (Madison, WI).

**Animals and treatments.** Male Sprague-Dawley rats and C57BL6 mice were obtained from Harlan Sprague-Dawley (Houston, TX) and Jackson Laboratory (Bar Harbor, ME), respectively. To induce ALD, both rats and mice (n = 4 in each group) were fed with Lieber-DeCarli liquid diet containing EtOH for 6 weeks followed by injection of LPS (Escherichia coli 0111:B4, ip in saline; 10 mg/kg body weight in rats and 1 mg/kg body weight in mice) and sacrificed 12-h post-LPS injection. The control animals (n = 4) were fed with an isocaloric maltose-dextrin diet. The exception to this protocol was in mice, where acclimatization to alcohol was for at least 1 week before the beginning of the experiments and provided humane care according to the institutional guidelines (University Laboratory Animal Care Committee) of Texas A&M University.

**Cell culture experiments.** All media and fetal bovine serum (FBS) were purchased from Mediatech, Inc. (Manassas, VA). HepG2 (ATCC, VA) and biliary epithelial cells (Dr. Yoshi Ueno, Japan) were grown at 37°C with 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) or DMEM/F12 (1:1) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator, respectively. A definite number of HepG2 or biliary epithelial cells (0.5 × 10⁶) were cultured and treated with EtOH (200mM), LPS (10 μg/ml), EtOH (200mM) plus LPS (10 μg/ml), and ACA (50μM) in DMEM or DMEM/F12 (1:1) with 5% FBS, respectively. The selection of the EtOH and LPS dose to induce OPN was based on previously published studies from this laboratory (Apte et al., 2005). After 24 h incubation, cells were harvested to carry out OPN induction studies.

**Preparation of total protein from treated liver and cultured cells.** Frozen liver tissues from rats and mice (~0.1 g) were homogenized in homogenization buffer (1% Triton X-100, 50mM NaCl, 10mM Tris, 1mM ethylenediaminetetraacetic acid, 1mM EGTA, 2mM Na vanadate, 0.2mM PMSF, 1mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) on ice with a homogenizer (Ika-Werke, Staffen, Germany). The cells were homogenized 3× for 10 s each, followed by a 30-s incubation on ice between each cycle. Homogenates were washed with TBS having 0.1% Tween20 and supernatants were collected and used for protein estimation. Total protein amount was calculated using Bio-Rad protein assay reagent (Bio-Rad, CA).

For cell culture experiments, cells were harvested by scraping following a rinse with ice-cold phosphate-buffered saline (PBS). Then, cells were collected and lysed in ice-cold lysis buffer (PBS having 0.2mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) on ice with quick-freeze thaw cycle (3×). Lysates were spun at 12,000 × g for 15 min at 4°C and supernatants were then collected and homogenized in buffer containing 25mM Tris (pH 8.3), 162mM glycine, and 0.1% SDS. Separated proteins were transferred onto nitrocellulose membrane (350 mA, 1.5 h) in transblotting buffer containing 25mM Tris (pH 8.3), 162mM glycine, 20% methanol, and 0.1% SDS. The membranes were then blocked with 6% milk in TBS buffer (1% Tween20, 0.1 g/ml) and incubated with a primary antibody (Santa Cruz), anti-goat secondary antibody (Santa Cruz) at 37°C for 1 h. After washing of membranes, the proteins were visualized with chemiluminescence reagent. The OPN antibody recognizes both the uncleaved form of OPN (~66 kDa), and the cleaved form of OPN (~32 kDa; Rüdiger and Feng, 1998). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control to ensure equal amount loading of proteins per well. Rabbit polyclonal anti-OPN primary antibody (Abcam, MA), anti-rabbit secondary antibody (Santa Cruz, CA), goat polyclonal anti-GAPDH primary antibody (Santa Cruz), anti-goat secondary antibody (Santa Cruz) were employed to detect and quantitate OPN protein.

**Preparation of total RNA.** Total RNA was isolated from liver tissue (in vivo) and cultured (HepG2 and biliary epithelial cells) by a 15 min homogenization (Ika-Werke, Staffen, Germany) with TRIzol reagent according to the manufacturer’s protocol (Invitrogen Corp., CA). Briefly, about 0.1 g of frozen liver tissue or 10 × 10⁶ cultured cells was homogenized in 1 ml of TRIzol on ice with a homogenizer. Homogenates were transferred into 1.5-mL tubes and 0.2 mL of chloroform was added into each tube. After vortexing for 15 s, mixtures were set in room temperature for 5 min and spun at 12,000 × g for 15 min at 4°C. Aqueous supernatant was carefully transferred into a new tube without disturbing the bottom phase and 0.5 mL of isopropanol was added and mixed with vortexing. Samples were set in room temperature for 10 min and centrifuged at 12,000 × g for 10 min at 4°C. After centrifugation, pellets were washed with 1 mL of 75% EtOH and centrifuged at 7000 × g for 8 min at 4°C. Pellets were then air-dried for 10 min and resuspended in RNase-free water.
RNA was dissolved completely by incubation at 55°C for 10 min. Total RNA concentration was determined by NanoDrop ND-1000 (Fisher, DE).

**RT-PCR assays.** Total RNA (100 ng) from all livers of treated rats, mice, or biliary epithelial cells was used for one step real-time quantitative SYBR Green RT-PCR in a total volume of 25 μl. Each reaction mixture contained 900nM forward and reverse primers each, 0.5 μl of RNase inhibitor and 0.125 μl of reverse transcriptase in SYBR Green master mix (Applied Biosystems, CA). ABI 7500 Real-Time PCR System (Applied Biosystems) was used, in which samples were denatured at 95°C for 10 min after pre-incubation of reactions at 50°C for 2 min. The thermal cycling step was for 40 cycles at 95°C for 15 s, and 40 cycles at 60°C for 1 min. Dissociation step (95°C for 15 s at 60°C for 1 min, and then 95°C for 15 s) was followed. The primers used in the RT-PCR reaction were as follow: mouse OPN forward 5'-TGC ACC CAG ATC CTA TAG CC-3', mouse OPN reverse 5'-CTC CAT GTG CAT CAT CAT CG-3', rat OPN forward 5'-TCA CCT CCC GCA TGA AGA G-3' and rat OPN reverse 5'-TCA GAC GCT GGG CAA CTG-3', PPAR-α forward 5'-CTG GTC AAG CTC AGG ACA CA-3', PPAR-α reverse 5'-AAG CTT CCG GAA CTC TTC TC-3', β-actin forward 5'-CCG TGA AAA GAT GAC CCA GAT C-3', and β-actin reverse 5'-CAC AGC CTC GTG GAT GGC TAC GT-3'. DNA contamination of the RNA extracts was tested with a no-RT reaction for each RNA sample. OPN and PPAR-α mRNA levels relative to the β-actin mRNA as a housekeeping gene were determined as previously described (Banerjee et al., 2006b).

**Transient transfection and luciferase assays.** Biliary epithelial cells were grown in DMEM/F-12 (1:1) containing 2.5% FBS. Duplicate samples of 0.5 × 10^5 cells in 12-well plates were transfected using Lipofectamine (Invitrogen, CA) with 0.3 μg of the 4×PPRE-luciferase reporter plasmid, 0.3 μg of pCMV-β-gal in DMEM/F-12 (1:1) without FBS (Chen et al., 1995). For PPAR-α overexpression, pcDNA3-PPAR-α was added to the transfection. Five hours following transfection, the cells were treated with various hepatotoxic agents in fresh medium with 2.5% FBS and incubated for 18 h. The cells were then washed with ice-cold PBS and harvested in reporter assay buffer (Promega, WI). Collected cells were lysed with quick freeze-thaw cycle (3×) and spun at 10,000 g from 10 min at 4°C. Supernatant was then subjected to the EtOH and EtOH plus LPS treatments showed enhanced expression of OPN protein (Figs. 1C and 1D). In contrast to rats, EtOH plus LPS treated mice did not show elevated OPN expression compared with the EtOH alone group.

Evaluation of OPN Induction by EtOH, LPS, EtOH plus LPS, and ACA In Vitro (Biliary Epithelium and HepG2 Cells)

The objective of this in vitro study was to assess if biliary epithelial cells and hepatocytes are responsive in vitro to OPN induction in vitro from Liver Homogenates of Rats and Mice in ALD Model

Similar to previous reports from this laboratory, OPN induction was noted in the rats treated with a combination of EtOH plus LPS (Banerjee et al., 2006a,b). EtOH plus LPS administration resulted in significant hepatic inflammation and liver damage (Banerjee et al., 2006a). In fact, EtOH alone resulted in nearly twofold elevation of OPN protein level and EtOH plus LPS combination resulted in more than fourfold elevations (Figs. 1A and 1B). LPS alone treated rats did not appear to show enhanced OPN expression. Similar to rats, mice subjected to the EtOH and EtOH plus LPS treatments showed enhanced expression of OPN protein (Figs. 1C and 1D).
induction by these hepatotoxic agents and to test whether these cell lines can be used to test OPN induction effect.

OPN protein was upregulated by all the hepatotoxic chemicals employed in biliary epithelial cells (Figs. 2A and 2B). Similar OPN induction was also observed within HepG2 cells although to a lesser degree (Figs. 2C and 2D). Interestingly, ACA, which is a major metabolite of EtOH, appeared to induce significant OPN both within biliary epithelium and HepG2 cells (Fig. 2).

**OPN mRNA Level In Vivo (Rats and Mice) and In Vitro (Biliary Epithelial Cells) following Hepatotoxic Chemical Treatment**

Consistent with OPN protein induction, OPN mRNA levels were significantly elevated in both rats (approximately fourfold; Fig. 3A) and mice (approximately fourfold; Fig. 3B) following EtOH treatment. Similar to *in vivo* studies, OPN mRNA expression was elevated following treatment with EtOH (twofold) and ACA (greater than twofold) within biliary epithelium (Fig. 3C).

**PPAR-α mRNA Expression In Vivo and In Vitro**

Because EtOH ingestion leads to hepatic inflammation and lipid accumulation and inactivation of hepatic PPAR-α (Crabb et al., 2012), OPN and PPAR-α expression was studied in ALD 293 cells.

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**FIG. 2.** OPN protein induction in the lysates of biliary epithelium and HepG2 cells *in vitro*. Biliary epithelial cells treated with EtOH (E), LPS (L), EtOH plus LPS (EL), and ACA were used to analyze OPN protein expression level by Western blot (A) and quantitated by densitometric analysis by normalizing with GAPDH protein (B). Similarly, HepG2 cells were treated with these agents for 24 h and the lysates were used to determine OPN expression level by Western blot (C) and quantitated by densitometric analysis by normalizing with GAPDH protein (D). OPN protein was detected around 66 kDa. Detail of the *in vitro* protocol is explained in methods section. All experiments were done in triplicates (*p* ≤ 0.05).

**FIG. 3.** OPN mRNA detection by RT-PCR from livers of EtOH-fed rats (A), EtOH-fed mice (B), and treated biliary epithelial cells (C). β-Actin was employed as an internal control for RT-PCR to ensure using equal amount of RNA. *Values significantly different from the controls. Data are expressed as mean ± SE, *p* ≤ 0.05.
mRNA level of PPAR-α was evaluated by real-time RT-PCR in our in vivo and in vitro models. PPAR-α mRNA level within the total liver homogenate was reduced in the EtOH-fed rat livers (~50%; Fig. 4A). Also, PPAR-α mRNA level was downregulated in the EtOH-fed mice compared with the control mice by ~50% (Fig. 4B). Similar to in vivo studies, biliary epithelial cells treated with EtOH and ACA in vitro showed lower PPAR-α mRNA level (>55% reduction) in both these groups compared with the control cells (Fig. 4C). Based on this and OPN induction data, there appears to be an opposite relationship between the expression patterns of PPAR-α (downregulation) and OPN (upregulation) expression both in in vivo and in vitro models following exposure to different hepatotoxic agents associated with EtOH ingestion.

**FIG. 4.** PPAR-α mRNA detection by RT-PCR from livers of EtOH-fed rats (A), mice (B), and treated biliary epithelial cells (C). β-Actin was employed as an internal control for RT-PCR to ensure using equal amount of RNA. *Values significantly different from the controls. Data are expressed as mean ± SE, p ≤ 0.05.

**PPAR-α Transcriptional Activity following Treatment of Biliary Epithelium with EtOH, LPS, EtOH plus LPS, and ACA**

To test whether the transcriptional activity of PPAR-α is also downregulated by these agents similar to their decreasing effects on PPAR-α mRNA level, PPRE reporter plasmid was employed (Tugwood et al., 1998). PPRE reporter plasmid containing luciferase as a reporter protein was transfected into biliary epithelial cells. Following transfection and hepatotoxic chemical treatments, luciferase assay showed that these agents reduced the activity of PPAR-α (Fig. 5A). EtOH, LPS, EtOH plus LPS, and ACA treatments resulted in reduction of PPAR-α transcriptional activity by approximately 50, 30, 70, and 30%, respectively, suggesting that the treatment reduced the transcriptional activity of PPAR-α besides their downregulating effects on PPAR-α mRNA expression (Figs. 4C and 5A).

**FIG. 5.** Transcriptional activity of PPAR-α was evaluated by luciferase assay after chemical treatment with the transfection of PPRE-luciferase reporter (A). PPAR-α activities were evaluated following PPAR-α overexpression (B) and PPAR-α overexpression plus exposure to PPAR-α agonist, bezafibrate (C). Luciferase activity was normalized by β-galactosidase assay. *Values significantly different from the controls. Data are expressed as mean ± SE, p ≤ 0.05.
**PPAR-α Overexpression Rescued the Reduced PPAR-α Activity**

To test whether PPAR-α is the limiting factor for the observed reduced PPAR-α mRNA level and the decreased transcriptional activity of PPAR-α following treatment of these agents, biliary epithelial cells were overexpressed with PPAR-α and cotransfected with PPRE-luciferase reporter plasmids within the treated biliary epithelial cells. Downregulated PPAR-α activity by these treatments was rescued by PPAR-α overexpression (Fig. 5B). Treatment of EtOH, LPS, EtOH plus LPS, and ACA increased transcriptional activity of PPAR-α by more than sevenfold, threefold, fourteenfold, and sixfold, respectively, suggesting that PPAR-α is the likely limiting factor for its transcriptional activation.

**PPAR-α Ligand Dependent Transcriptional Activity**

Because downregulated PPAR-α activity by the treatment of EtOH, LPS, EtOH plus LPS, and ACA was rescued by exogenous PPAR-α overexpression (Fig. 5B), biliary epithelial cells were exposed to a relatively well-known PPAR-α ligand, bezafibrate to test whether transcriptional activity of PPAR-α is elevated by exposure to PPAR-α agonist. Following treatment with hepatotoxic chemicals, cells were exposed to bezafibrate (100μM) after cotransfection with PPRE reporter plasmid and PPAR-α overexpression plasmid. Bezafibrate treatment significantly elevated PPAR-α activity in vitro in EtOH treated group (Fig. 5C). Bezafibrate treated EtOH group showed 1.8-fold higher transcriptional activity of PPAR-α, although such increased PPAR-α activity was not noted in the other groups (Fig. 5C).

**Hepatic Injury as Evaluated by Hematoxylin and Eosin Stained Liver Sections in Wild-Type and OPN−/− Mice following EtOH, LPS, and EtOH plus LPS Treatment**

To further investigate the opposite relationship noted between OPN and PPAR-α in ALD model in vivo, and to assess the effect of OPN in causing liver injury and inflammation, OPN−/− mice model was also utilized in this study. OPN−/− mice were fed with Lieber-DeCarli liquid diet for 6 weeks similar to the wild-type mice. Wild-type mice experienced significant lipid accumulation within the hepatic parenchyma following EtOH feeding (Fig. 6C). Also, OPN−/− mice showed hepatic lipidosis although the degree of fat accumulation appeared to be higher than the wild-type mice (Fig. 6D). LPS alone treatment did not cause the significant hepatic in jury in wild-type and OPN−/− mice (Figs. 6E and 6F). However as anticipated, EtOH plus LPS treated wild-type mice experienced higher hepatic neutrophilic inflammation (Fig. 6G). Compared with the wild-type mice, OPN−/− experienced significantly lesser degree of inflammation following EtOH plus LPS treatment (Fig. 6H).

**Relationship between PPAR-α and OPN in the Wild-Type and OPN−/− Mice following EtOH Treatment**

Figure 7 shows that PPAR-α mRNA level was reduced by EtOH feeding and LPS injection in wild-type mice as expected. In contrast, PPAR-α mRNA level in OPN−/− mice were not reduced by EtOH plus LPS treatment.

**DISCUSSION**

Consistent with our previous reports in a rat model of ALD, this study identified an induction of hepatic OPN in both rats and mice where there was hepatic inflammation and injury. Previously we have shown significant hepatic steatosis and steatohepatitis in an EtOH and LPS combination rat model of ALD (Apte et al., 2005, Banerjee et al., 2006a,b). Compared with rats, mice require significantly lesser amount of EtOH and shorter duration of exposure to EtOH to induce hepatic pathology. Based on the results in this study, species differences appear to exist in the degree of OPN induction between rats and mice during the administration of EtOH plus LPS combination. The combination group in rats resulted in significantly higher OPN induction compared with the mice, where LPS administration did not further enhance the level of OPN induction. This discrepancy can be partly attributed to the higher mice susceptibility to EtOH compared with the rats. In fact, the EtOH plus LPS combination is required to induce significant hepatic pathology in rats, whereas only EtOH alone is sufficient to cause comparable hepatic pathology in mice. Nonetheless, because we have identified OPN to be an important indicator of hepatic inflammation in vivo, this study was conducted to assess if OPN can also be induced in vitro. Although animal models are very informative to investigate liver diseases such as EtOH-mediated liver injury, it requires more than 6 weeks to induce the liver pathology and to evaluate the relationship between hepatobiliary OPN expression and liver injury. HepG2 and biliary epithelial cells were utilized to evaluate OPN expression following various agents associated with EtOH ingestion, because OPN has been reported to be expressed mainly from biliary epithelium and hepatocytes in vivo (Banerjee et al., 2006a, b). Although EtOH and a combination of EtOH and LPS treatment to rats and mice showed OPN induction, LPS alone at the employed dose did not induce significant OPN expression in vivo.

Similar to the in vivo data, in vitro experiments showed OPN induction by the treatment with EtOH, EtOH plus LPS, and ACA. These results suggest that OPN induction can be achieved in vitro by EtOH and its related metabolites and that OPN induction can be potentially employed to predict hepatic inflammation in vitro. A discrepancy noted in this study is the ability of LPS to cause OPN induction in vitro and not in vivo. This may be related to the dose of LPS employed in vivo, where LPS alone did not cause significant hepatic inflammation compared with EtOH plus LPS. With respect to the in vitro model, a potential concern that can be raised is its usefulness to mimic the in vivo ALD model. It should be noted that EtOH was administered daily for several weeks in vivo in both rats and mice versus a single dose in vitro. Although significant
FIG. 6. Histopathologic evaluation of hematoxylin and eosin stained liver sections in wild-type and OPN$^{-/-}$ mice following EtOH, LPS, and EtOH plus LPS treatment; control (A and B), EtOH (C and D), LPS (E and F), and EtOH plus LPS (G and H) in wild-type and OPN$^{-/-}$ mice. Circled areas indicate hepatic inflammation as denoted by neutrophilic infiltration. Magnification ×40. Details of the in vivo protocol are explained in methods section.
lipid accumulation is not noted following EtOH exposure in vitro, oxidant stress (elevated CYP2E1; data not shown) associated with ALD is a significant finding in this model. In fact, oxidant stress associated with CYP2E1 following EtOH exposure in HepG2 cells has been previously reported (Cederbaum et al., 2001).

In addition to hepatobiliary OPN induction associated with concurrent hepatic inflammation, lipid accumulation (steatosis) in the liver is a concurrent pathologic finding associated with ALD. PPARα appears to be crucial in the pathogenesis of hepatic steatosis which occurs prior to hepatic inflammation during chronic alcohol consumption (Crabb et al., 2004; Ramaiah et al., 2004). In fact, it is reported that PPARα agonist treatment in EtOH-fed mice reverses the hepatic lipid accumulation in mice (Fischer et al., 2003). Interestingly, a recent study showed that PPARα agonist suppresses OPN expression in macrophages in a model of type-2 diabetes (Nakamachi et al., 2007). Because OPN is increased and PPARα is decreased in the in vivo model of ALD, we investigated the PPARα expression levels in EtOH-fed rats and mice where significant OPN induction was noted. PPARα mRNA level was significantly reduced by EtOH feeding in both rats and mice.

Consistent with in vivo data, biliary epithelial cells treated with EtOH and ACA downregulated PPARα mRNA level. Because PPARα mRNA level was downregulated by EtOH and ACA treatment, transcriptional activity of PPARα was determined using PPRE-luciferase reporter plasmid in biliary epithelial cells. Although PPARα activity was downregulated by treatment with these agents, it is not clear whether reduced PPARα activity is solely because of reduced PPARα expression level or due to downregulation of endogenous PPARα ligand levels. PPARα needs a coactivator, RXR and its ligand such as free fatty acids to function as a transcriptional factor in cells (Zoete et al., 2007). To test whether PPARα is limiting factor in reduced transcriptional activity by treatment of EtOH, LPS, EtOH plus LPS, and ACA, PPARα over-

FIG. 7. PPARα mRNA levels in wild-type and OPN−/− mice treated with EtOH plus LPS were determined by real-time RT-PCR. The values were normalized with β-actin—the housekeeping gene. *Values significantly different from the controls. Data are expressed as mean ± SE, *p ≤ 0.05. Details of the in vitro protocol are explained in methods section and previous reports.

expression experiments revealed elevated PPARα transcriptional activity. These results show that PPARα may be a limiting factor in the reduced PPARα activity by treatment with these hepatotoxic chemicals.

To test if PPARα agonist will further elevate PPARα activity after PPARα overexpression, exposure to PPARα agonist bezafibrate showed enhanced PPARα activity as evidenced by the luciferase assay in only the EtOH treated group. Interestingly, PPARα agonist did not increase PPARα activity in other groups. Additional investigation with different PPARα agonists, their dose-response, and different cell lines are needed to confirm these findings.

Most of the data presented in this study that implicates the relationship between hepatobiliary OPN and PPARα expression during hepatotoxic chemical treatment are mostly correlative. To further identify a relationship between these two proteins during ALD, OPN−/− studies yielded an interesting finding. Absence of OPN in the knock out mice did not decrease the PPARα mRNA level following EtOH feeding in contrast to the wild-type mice, suggesting the influence of OPN on PPARα reduction. Potential relationship between OPN and PPARα in the liver is summarized and hypothesized in Figure 8. Treatment with EtOH and ACA induce OPN and reduced PPARα expression. Although OPN is involved in hepatic inflammation, PPARα

FIG. 8. Schematic of the potential relationship between hepatic OPN and PPARα expression. The data presented in this study provide evidence for the OPN-inducing effect of EtOH and ACA on hepatic OPN induction which is known to result in hepatic inflammation and injury during ALD. Concurrent to OPN induction (EtOH and ACA), there is an inhibition of PPARα expression leading to increased hepatic lipid accumulation, inflammation and liver injury. The correlative relationship between OPN upregulation and PPARα inhibition during ALD is supported by OPN−/− studies where the lack of OPN does not result in PPARα downregulation. On the contrary, PPARα upregulation did not result in the decreased OPN expression in vitro.
is known to play an important role in hepatic lipid metabolism (Crabb et al., 2004). Also, OPN−/− mice showed lesser hepatic inflammation following EtOH consumption compared with wild-type, although the degree of fat accumulation appeared to be significantly higher in the EtOH alone treated OPN−/− mice than the wild-type. From these results, we hypothesize that OPN and PPAR-α may be operating in an opposite fashion in ALD models both in vivo and in vitro.

If OPN is influencing PPAR-α expression, another scenario was tested to assess if PPAR-α overexpression can change OPN level in vitro. Biliary epithelial cells were transfected with serial dilution of PPAR-α overexpression plasmid and cell lysate was measured for OPN levels following different chemical treatment. OPN expression was not influenced by PPAR-α overexpression suggesting that OPN expression has a negative effect on PPAR-α expression but not vice versa (unpublished data). These results are somewhat different with the reports of Nakamachi et al. (2007) who showed that PPAR-α agonist suppressed OPN production. This difference can be attributed to the cell-type and the experimental design employed to enhance PPAR-α activity. Future experiments aimed at either increasing PPAR-α activity such as by different PPAR-α agonists (such as bezafibrate or clofibrate) or by knocking down PPAR-α (knock out mice) and evaluating OPN expression should provide additional information on the influence of PPAR-α on OPN expression. At least based on our studies, it is likely that OPN can be directly targeted to decrease inflammation by mechanisms that inhibit OPN expression rather than through PPAR-α. Additional investigations are needed in the future to delineate the precise mechanism of the regulation of OPN and PPAR-α expressions in EtOH and ACA treated models to clarify this complex relationship.

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