Suppression of the Pregnane X Receptor during Endoplasmic Reticulum Stress Is Achieved by Down-Regulating Hepatocyte Nuclear Factor-4α and Up-Regulating Liver-Enriched Inhibitory Protein

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ABSTRACT

Endoplasmic reticulum (ER) stress is recognized as a common theme in the development of metabolic syndrome and other diseases. Chronic liver diseases develop ER stress and also show decreased capacity of drug metabolism. The pregnane X receptor (PXR) is a master regulator of genes involved in drug elimination. This study was performed to determine whether ER stress condition decreases the expression of PXR and whether the decrease alters the induction of cytochrome P450 3A4 (CYP3A4). Human primary hepatocytes and HepG2 cell line (human hepatocellular carcinoma) were treated with brefeldin A and thapsigargin, 2 well-established ER stressors. Without exceptions, both stressors significantly decreased the expression of PXR. The decrease led to reduced induction of CYP3A4. Reporter dissection study, electrophoretic mobility shift assay, and chromatin immunoprecipitation located in the PXR promoter region 2 adjacent elements recognized by hepatocyte nuclear factor-4α (HNF-4α) and cytidine-cytidine-adenosine-adenosine-thymidine enhanced binding proteins (C/EBPs), respectively. Additional studies demonstrated that HNF-4α was down-regulated during ER stress but the expression of C/EBPβ varied depending on a particular form of C/EBPβ. Liver-enriched activator protein (LAP) was down-regulated but liver-enriched inhibitory protein (LIP) was highly induced. Nevertheless, over-expression of HNF-4α or LAP restored the expression of PXR. Interestingly, the very same sequence also responded to interleukin-6 (IL-6), and primary hepatocytes treated with thapsigargin significantly increased the level of IL-6 mRNA. These findings establish a functional interconnection between ER stress and signaling of proinflammatory cytokines in the regulation of PXR expression.

Key words: PXR; ER stress; CYP3A4; proinflammatory cytokines; STAT3

The endoplasmic reticulum (ER) is an organelle involved in diverse cellular functions including protein synthesis/transportation, membrane generation, calcium concentration regulation, and xenobiotic metabolism (Johnson et al., 2013; Li et al., 2012). Therefore, ER homeostasis is critical in maintaining the overall cellular functions. On the other hand, many factors such as oxidative stress disrupt ER homeostasis, leading to ER stress (Adolph et al., 2012; Cali et al., 2011; Xu and Zhu, 2012). While the precise mechanisms of ER stress remain to be determined, one of the outcomes is the accumulation of unfolded proteins in the ER (Benbrook and Long, 2012; Haeri and Knox, 2012). The unfolded protein response (UPR), occurring at the initial stage of ER stress, is triggered to slow down protein synthesis, improve protein folding capacity, and enhance degradation of unfolded proteins (Lin et al., 2012). Nevertheless, persistent ER stress has been linked to the development of various conditions such as type 2 diabetes and chronic liver diseases (Back and Kaufman, 2012; Bock and Bock-Hennig, 2010; Pagliassotti, 2012; Santoro et al., 2007).
The liver is the largest internal organ and plays the primary role in drug metabolism (Bock and Bock-Hennig, 2010; Santoro et al., 2007; Villarroya et al., 2010). The prevalence of hepatic dysfunction is high and it affects more than 10% of Americans (Liver foundation, 2009). Worldwide, liver cancer and chronic liver diseases are the seventh leading cause of death (IPA, 2007). Many liver diseases are accompanied with ER stress and exhibit decreased capacity of drug metabolism and detoxication. Steatotic livers, for example, were found to have significant decreases in the activity of cytochrome P450 3A4 (CYP3A4) (Kolwankar et al., 2007), the most robust catalytic enzyme in the oxidative metabolism of drugs and other xenobiotics. In cultured primary hepatocytes, lipid loading significantly decreased the expression of CYP3A4 (Donato et al., 2007). Furthermore, livers from diabetic patients showed significantly lower expression of CYP3A4 (Dostalek et al., 2011).

The expression of CYP3A4 is regulated by several major transcription factors. Among these proteins, the pregnane X receptor (PXR) has been established to play the primary role (Ihunnah et al., 2012). PXR binds to PXR response elements that contain a half-site AG(G/T)TCA or related sequence. We and other investigators have functionally characterized 4 PXR elements in the CYP3A4 gene and some of the elements operate in a coordinate manner (Goodwin et al., 1999; Liu et al., 2008; Song et al., 2004; Torigabe et al., 2009). While PXR regulates the expression of CYP3A4 and many other drug-elminating genes, we have demonstrated that the expression of PXR was altered by drugs and disease mediators (Ma et al., 2005; Shi et al., 2010; Yang et al., 2010). Importantly, the expression of PXR directly affects CYP3A induction. Dexamethasone, a synthetic glucocorticoid, induces PXR and synergistically induces CYP3A (Shi et al., 2010). Likewise, clofibrate, the lipid-lowering agent, causes super-induction of CYP3A23 (Ma et al., 2005). Conversely, interleukin-6 (IL-6), a proinflammatory cytokine, decreases PXR expression and reduce CYP3A4 induction (Yang et al., 2010).

This study was performed to determine whether ER stress condition decreases the expression of PXR and whether the decrease alters the induction of CYP3A4. Brefeldin A (BFA) and thapsigargin (Thaps), 2 well-established ER stressors (Nickel et al., 2010; Ri et al., 2012; Salido et al., 2009), significantly decreased the expression of PXR in both primary hepatocytes and HepG2 cell line (human hepatocellular carcinoma). The decrease led to reduced induction of CYP3A4. The decrease of PXR expression was achieved by transcriptional repression via 2 adjacent elements recognized by hepatocyte nuclear factor-4α (HNF-4α) and cytidine-cytidine-adenosine-adenosine-thymidine enhanced binding proteins (C/EBPs), respectively. Over-expression of either protein restored the expression of PXR. Interestingly, the adjacent elements also responded to IL-6, suggesting a functional interconnection between ER stress and signaling of proinflammatory cytokines.

MATERIALS AND METHODS

Chemicals and supplies. IL-6 and Thaps were from R&D Systems (Minneapolis, Minnesota). BFA, Hanks balanced salt solution, and the antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Sigma (St Louis, Missouri). Dulbecco’s Modified Eagle’s Medium (DMEM) and high fidelity Platinum Taq DNA polymerase were from Life Technology (Carlsbad, California). The antibodies against HNF4α or C/EBPβ were from Abcam Inc (Cambridge, Massachusetts). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce (Rockford, Illinois). Plated human primary hepatocytes were obtained from the Liver Tissues Procurement and Distribution System (University of Minnesota) or CellzDirect (Pittsboro, North Carolina). Nitrocellulose membranes were from Bio-Rad (Hercules, California). Expression constructs were from OriGene Technologies Inc (Rockville, Maryland). Unless otherwise specified, all other reagents were purchased from Fisher Scientific (Fair Lawn, New Jersey).

Reverse transcription-quantitative polymerase chain reaction. Total RNA (1 µg) was subjected to the synthesis of the first strand cDNA as described previously (Xiao et al., 2013). cDNAs were then diluted 8 times and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was conducted with TaqMan Gene Expression Assay (Applied Biosystems, Foster City, California). The TaqMan probes were: PXR, Hs00243666_m1; HNF4α, Hs00230853_m1; C/EBPβ, Hs00942496_s1; IL-6, Hs00985639_m1; and GAPDH, 4352934E; and RNA polymerase II, Hs00221877_m1. The normalization of TaqMan qPCR was performed presently based on the signal of GAPDH mRNA and selective samples were analyzed for the level of RNA polymerase II to confirm the normalization. The PCR amplification was conducted in a total volume of 20 µl containing universal PCR master mixture (10 µl), gene-specific TaqMan assay mixture (1 µl), and cDNA template (6 µl). The mRNA levels were normalized according to the level of GAPDH and the normalization of selected samples was confirmed based on the signal of RNA polymerase II. Amplification and quantification were done with the Applied Biosystems 7500 Real-Time PCR System.

Reporter constructs and cotransfection assays. PXR promoter reporters were prepared to contain various lengths of PXR genomic sequence. All promoter reporters were subcloned from the FXR-1285-Luc reporter through Mlu I and Xho I sites. While PXR regulates the expression of CYP3A4 and many other drug-elminating genes, we have demonstrated that the expression of PXR was altered by drugs and disease mediators. The expression of CYP3A4 is regulated by several major transcription factors. Among these proteins, the pregnane X receptor (PXR) has been established to play the primary role (Ihunnah et al., 2012). PXR binds to PXR response elements that contain a half-site AG(G/T)TCA or related sequence. We and other investigators have functionally characterized 4 PXR elements in the CYP3A4 gene and some of the elements operate in a coordinate manner (Goodwin et al., 1999; Liu et al., 2008; Song et al., 2004; Torigabe et al., 2009). While PXR regulates the expression of CYP3A4 and many other drug-elminating genes, we have demonstrated that the expression of PXR was altered by drugs and disease mediators (Ma et al., 2005; Shi et al., 2010; Yang et al., 2010). Importantly, the expression of PXR directly affects CYP3A induction. Dexamethasone, a synthetic glucocorticoid, induces PXR and synergistically induces CYP3A (Shi et al., 2010). Likewise, clofibrate, the lipid-lowering agent, causes super-induction of CYP3A23 (Ma et al., 2005). Conversely, interleukin-6 (IL-6), a proinflammatory cytokine, decreases PXR expression and reduce CYP3A4 induction (Yang et al., 2010).

This study was performed to determine whether ER stress condition decreases the expression of PXR and whether the decrease alters the induction of CYP3A4. Brefeldin A (BFA) and thapsigargin (Thaps), 2 well-established ER stressors (Nickel et al., 2010; Ri et al., 2012; Salido et al., 2009), significantly decreased the expression of PXR in both primary hepatocytes and HepG2 cell line (human hepatocellular carcinoma). The decrease led to reduced induction of CYP3A4. The decrease of PXR expression was achieved by transcriptional repression via 2 adjacent elements recognized by hepatocyte nuclear factor-4α (HNF-4α) and cytidine-cytidine-adenosine-adenosine-thymidine enhanced binding proteins (C/EBPs), respectively. Over-expression of either protein restored the expression of PXR. Interestingly, the adjacent elements also responded to IL-6, suggesting a functional interconnection between ER stress and signaling of proinflammatory cytokines.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay (EMSA) experiment was performed as described previously (Liu et al., 2008; Yang et al., 2012). Nuclear extracts of HepG2 cells treated with Thaps (10 nM) for 24 h were prepared with the nuclear and cytoplasmic extraction kit (Pierce, Rockford, Illinois). The sense and antisense oligonucleotides (Table 1) were annealed by heating at 94°C for 5 min followed by gradually cooling to room temperature. The sense strand was...
were precleared for 2 h at 4°C. Cross-linking was terminated with glycine (final concentration 100 mM) for 24 h, washed and underwent cross-linking for 1 h with thapsigargin (Thaps, 10 nM) for 24 h, washed and underwent cross-linking for 1 h with thapsigargin (Thaps, 10 nM) for 24 h, washed and underwent cross-linking for 1 h with thapsigargin (Thaps, 10 nM) for 24 h, washed and underwent cross-linking for 1 h with thapsigargin (Thaps, 10 nM) for 24 h, washed and underwent cross-linking for 1 h with thapsigargin (Thaps, 10 nM) for 24 h.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) experiment was performed, essentially described previously (Cali et al., 2011; Johnson et al., 2013).

RESULTS

Down-regulation of PXR by ER Stressors

ER stress is a phenomenon in various chronic diseases and many PXR-target genes are down-regulated by disease conditions associated with ER stress (Cali et al., 2011; Johnson et al., 2013).

To test whether the expression of PXR itself is decreased during ER stress, HepG2 cells were treated with Thaps and BFA. Thaps induces ER stress by depleting calcium in the ER (Saldio et al., 2009), whereas BFA by retrograde-transporting proteins from the Golgi apparatus to the ER (Nichel, 2010). As shown in Figure 1A, treatment with either stressor significantly decreased the level of PXR mRNA. BFA was slightly more potent than Thaps (Fig. 1A).

Next, we determined the decrease of PXR mRNA as a function of the amount of Thaps. As shown in Figure 1B, Thaps at 1 nM caused a 20% decrease of PXR mRNA and at 10 nM caused a 40% decrease. Further increased concentrations of Thaps up to 250 nM caused only a 10% additional decrease (Fig. 1B). To ascertain the cellular ER stress level, semi-quantitative PCR was performed to detect the presence of spliced X-box binding protein 1 (XBP1 gene) mRNA, a widely used marker for ER stress (Ri et al., 2012). As shown in Figure 1B (bottom), little spliced XBP1 mRNA was detected in cells treated with solvent or 1 nM Thaps. Comparable levels of spliced and non-spliced XBP1 mRNA were detected in cells treated at 10 nM (Fig. 1B). In contrast, cells treated at 25 or 250 nM exhibited the presence of spliced XBP1 mRNA only. To gain in vivo relevance, primary hepatocytes were treated with Thaps, and the expression of PXR was determined.

As shown in Figure 1C, Thaps decreased PXR at both mRNA and protein levels, and the decrease was even greater than that in HepG2 cells (Figs. 1A and 1B).

Transcriptional Repression of PXR by Thaps

The decreases in PXR mRNA pointed to 2 possibilities: ER stressors enhanced PXR mRNA degradation and/or reduced PXR transactivation. To shed light on the second possibility, various PXR reporters containing the promoter or along with up-stream regulatory sequences at varying length were tested for the repression in response to Thaps. As shown in Figure 2A, all PXR reporters, compared with the vector control, were significantly repressed. However, the reporter PXR-56Luc, compared with PXR-104Luc, was repressed to a significantly less extent, suggesting that the DNA segment from –104 to –56 nt is critical for the repression. Based on element prediction with computer program, this DNA segment contains an HNF4a and a C/EBP binding site. These 2 elements are spaced by 3 nt (Fig. 2A).

It should be noted that as many as 5 transcription start sites (filled triangles in Fig. 2A) are located in the PXR promoter region (Kurose et al., 2005; Tompkins et al., 2008; Zhang et al., 2001).

Table 1. Sequences of Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Native promoter</td>
<td></td>
</tr>
<tr>
<td>reporters (numbered</td>
<td></td>
</tr>
<tr>
<td>according to Kurose</td>
<td></td>
</tr>
<tr>
<td>et al. [2005])</td>
<td></td>
</tr>
<tr>
<td>FXR-128-Mul</td>
<td>5’-cttggctatgcccagggaggctatggc-3’</td>
</tr>
<tr>
<td>FXR-505-Mul</td>
<td>5’-gttgctacgaatgcccagggaggctatggc-3’</td>
</tr>
<tr>
<td>FXR-204-Mul</td>
<td>5’-attggctatgcccagggaggctatggc-3’</td>
</tr>
<tr>
<td>FXR-104-Mul</td>
<td>5’-attgtacgaatgcccagggaggctatggc-3’</td>
</tr>
<tr>
<td>FXR-56-Mul</td>
<td>5’-gttggctatgcccagggaggctatggc-3’</td>
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<tr>
<td>FXR-14-BamH1</td>
<td>5’-gagaatgctatgcccagggaggctatggc-3’</td>
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<td>Element reporters</td>
<td></td>
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</tr>
<tr>
<td>FXR-HNF4a mutant</td>
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</tr>
<tr>
<td>FXR-C/EBP mutant</td>
<td>5’-gtatgctatgcccagggaggctatggc-3’</td>
</tr>
<tr>
<td>FXR-double mutant</td>
<td>5’-gtatgctatgcccagggaggctatggc-3’</td>
</tr>
<tr>
<td>EMSA</td>
<td></td>
</tr>
<tr>
<td>FXR-HNF4a</td>
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<tr>
<td>FXR-HNF4a (mutant)</td>
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<tr>
<td>FXR-C/EBP mutant</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>Element sense</td>
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<tr>
<td>Element reverse</td>
<td>5’-gggattatgcccagggaggctatggc-3’</td>
</tr>
<tr>
<td>Non-element sense</td>
<td>5’-gggattatgcccagggaggctatggc-3’</td>
</tr>
<tr>
<td>Non-element reverse</td>
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</tr>
<tr>
<td>Semi-quantitative</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
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<td>XBP1-sense</td>
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</tr>
<tr>
<td>XBP1-reverse</td>
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</tr>
<tr>
<td>GAPDH-reverse</td>
<td>5’-ggggattatgcccagggaggctatggc-3’</td>
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32 cycles at 94°C for 30 s, 58°C for 30 s, and 68°C for 60 s. A 3-min initial denaturation was performed.

Other analyses. Protein concentrations were determined with BCA assay (Pierce) based on albumin standard. Western blotting was performed as described previously (Shi et al., 2011) and the preparation of the antibody against PXR was described elsewhere (Sachdeva et al., 2003). Data are presented as mean ± SD of at least 3 separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Statistical significance between 2 means was made according to 1-way ANOVA followed by a DUNCAN’s multiple comparison test (P < 0.05).
Specifically, the wild-type reporter was repressed by 45%, the showed less repression by Thaps than the wild-type reporter. All mutant reporters (disrupted HNF4 element) then reporter activity was determined. As shown in Figure 2B, HepG2 cells were transfected with the wild-type or a mutant reporters were tested for the abolished response to Thaps. The resultant element reporters were prepared to contain this segment or (Fig. 4B). Incubation with extracts from Thaps-treated cells produced a new shifted band (probably bound by LIP) and decreased the intensities of 2 shifted bands (arrowed in Fig. 4A). Incubation with the C/EBP site and the other the C/EBP a form of C/EBP b, treated with Thaps, and detected for the level of PXR mRNA. The C/EBP element is known to support the binding for multiple proteins including LAP, a major form of C/EBP b (Tsukada et al., 2011). Importantly, C/EBP b proteins have been reported to alter their functionality during ER stress (Arensdorf and Rutkowski, 2013; Li et al., 2008). As shown in Figure 2C, transfection of HNF4a or LAP reversed the decrease of PXR mRNA in Thaps-treated cells (columns 3 and 4 vs column 2). The transfection experiment pointed to 2 important conclusions: both HNF4a and LAP are positive regulators (eg, transactivators) of the PXR gene and they can function independently of each other.

Effect of Thaps on the Expression of HNF4a and C/EBP (LAP and LIP) Next we tested whether Thaps decreases PXR expression by down-regulating HNF4a and C/EBP b. Cells were treated with Thaps and the expression of HNF4a and C/EBP b was determined by RT-qPCR and Western blotting. As shown in Figure 3A, Thaps surprisingly caused a 9- to 11-fold induction of C/EBP b mRNA. It has been well established that C/EBP b mRNA produces several in-frame polypeptides including LAP* (38 kDa), LAP (35 kDa), and liver-enriched inhibitory protein (LIP: 20 kDa) (Tsukada et al., 2011). Importantly, these polypeptides are functionally distinct with LAP* and LAP being activators and LIP being an inhibitor. To determine whether the increased C/EBP b mRNA by Thaps differentially increases these polypeptides, Western blotting was performed. As shown in Figure 3A (Right), little changes were detected on the levels of LAP and LIP*. In contrast, LIP was markedly increased. These results established that induction of C/EBP b mRNA by Thaps increased the production of the inhibitor LIP but not the activators LAP* and LAP. The level of HNF4a mRNA, in contrast to the level of C/EBP b mRNA, was significantly decreased by Thaps (Fig. 3B) and the decrease was less with prolonged treatment. The 12 h time-point showed a 60% decrease whereas the 24 h time-point showed a 40% decrease (Fig. 3B). Consistent with the decrease in HNF4a mRNA, the level of HNF4a protein was drastically decreased (Fig. 3B).

Occupancy of the PXR Promoter by HNF4a and C/EBP b The reporter and expression studies collectively suggested that the PXR promoter is targeted by HNF4a and C/EBP b. To directly test this possibility, EMSA and ChIP experiments were performed. The EMSA experiment was performed with 2 probes: one containing the putative HNF4a site and the other the C/EBP site. As shown in Figure 4A, incubation with the HNF4a probe led to the detection of a major shifted band (Fig. 4A). The intensity of this band was weaker when incubation was performed with nuclear extracts from Thaps-treated cells, consistent with the fact that Thaps down-regulated HNF4a. This band was competed by non-labeled probe and abolished by the antibody against HNF4a. Addition of the antibody also intensified the band on the top of the gel (Fig. 4A). Incubation with the C/EBP b probe, on the other hand, led to the detection of several bands (Fig. 4B). Incubation with extracts from Thaps-treated cells produced a new shifted band (probably bound by LIP) and decreased the intensities of 2 shifted bands (arrowed in column 3). All shifted bands except the top one were competed by non-labeled probe but not by the corresponding non-labeled probe.
probe with disrupted C/EBP-binding site. Nonetheless, the putative LAP- and LIP-binding bands were abolished by the antibody against C/EBPβ (Fig. 3).

The EMSA experiment established that the PXR promoter contained HNF4α and C/EBP-binding site. Next we tested whether both proteins occupy the PXR promoter. ChIP experiment was performed in cells treated with solvent or Thaps. In addition to HNF4α and C/EBP element-containing segment, a segment of the PXR gene containing either element was subjected to PCR-amplification as a control. As shown in Figure 4C, PCR detected the amplification of both segments with input DNA. However, PCR detected the HNF4α-C/EBP but not the control segment with ChIPed-DNA. The amplification was observed with ChIPed DNA from control but not Thaps-treated cells (Fig. 4C). It should be noted that preimmune IgG for ChIP experiment did not yield any amplification.

Interconnection between Thaps and IL-6 in the Suppression of PXR
We have previously showed that PXR was down-regulated by the proinflammatory cytokine IL-6 (Yang et al., 2010). To
determine whether Thaps and IL-6 use similar genomic sequence in the down-regulation, HepG2 cells were transfected with various PXR reporters, treated with IL-6 as shown, and detected for luciferase activity. BFA, another commonly used ER stressor, was also included in this study. As predicted, both IL-6 and BFA produced a similar responding pattern as Thaps among these reporters (Figs. 2A and 5A). Two additional experiments were performed to shed light on the mechanistic connection between ER stress and IL-6. Firstly, the suppression of PXR by IL-6 and Thaps was determined as a function of the time of treatment. Secondly, the expression of IL-6 was determined in Thaps-treated primary hepatocytes. As shown in Figure 5B, both IL-6 and Thaps significantly decreased PXR mRNA. However, the decrease by IL-6 occurred sooner than that by Thaps (Fig. 5B). We next tested whether human primary hepatocytes treated with Thaps actually support the induction of IL-6. As shown in Figure 5C, treatment with Thaps significantly increased IL-6 mRNA (Fig. 5C).

**Effect of ER Stress on CYP3A4 Induction**

The enhanced production of IL-6 by Thaps suggested that IL-6 is a contributor to Thaps-mediated down-regulation of PXR. It is well established that signal transducer and activator of transcription-3 (STAT3) supports the activity of IL-6 (Bode et al., 2012). We next tested whether Z-guggulsterone, a blocker of STAT3 (Leeman-Neill et al., 2009), antagonizes Thaps in down-regulating PXR. On the other hand, Z-guggulsterone is a known antioxidant (Chen et al., 2012), therefore, emodin was included in this study as a control for antioxidant property (Shia et al., 2010). HepG2 cells were treated with Thaps, Z-guggulsterone, emodin, or in combination, and then the level of PXR mRNA was determined. As expected, Thaps significantly decreased PXR mRNA (Fig. 6A). The decrease, however, was almost completely reversed by Z-guggulsterone but not by emodin. It should be noted that Z-guggulsterone and emodin alone showed no effect on the level of PXR mRNA (Fig. 6A).

Next we tested whether overexpression of PXR itself reverses the effect of Thaps in terms of the induction of CYP3A4. Both transfected and non-transfected HepG2 cells were used, and the transfection was performed with a PXR expression construct or the corresponding vector. The cells were treated with Thaps, rifampicin, or both for 24 h and analyzed for the mRNA level of CYP3A4, a prototypical target of PXR (Klein and Zanger, 2013).
The results were expressed as fold of induction. As shown in Figure 6B, Thaps significantly decreased the induction of CYP3A4 in both vector- and nontransfected cells. However, the decrease was reversed in PXR transfected cells.

**DISCUSSION**

ER stress is recognized as a common theme in the development of metabolic syndrome and other diseases (Back and Kaufman, 2012; Flamment et al., 2012; Johnson et al., 2013; Lin et al., 2012; Pagliassotti, 2012) and emerging evidence has pointed to decreased capacity of metabolism in liver diseases associated with ER stress (Flamment et al., 2012; Pagliassotti, 2012). PXR is a master regulator of genes in xenobiotic elimination. In this study, we have shown that Thaps and BFA, 2 well-characterized ER stressors significantly decreased the expression of PXR. The decrease was mediated through transcriptional repression and led to reduced induction of CYP3A4, a prototypical target gene of PXR (Klein and Zanger, 2013). The decrease of PXR expression by Thaps was reversed by Z-guggulsterone, an active ingredient of the hypolipidemic herb guggul (Yang et al., 2012).

It is likely that the reversal by Z-guggulsterone was achieved by blocking STAT3 activity. Several lines of evidence support this possibility. Firstly, Z-guggulsterone is an antioxidant and many antioxidants reportedly protect against ER stress (Ding et al., 2013; Li et al., 2012), however, emodin (an antioxidant) showed no reversal activity on the Thaps-mediated downregulation of PXR (Fig. 6A, Harlev et al., 2012), excluding an involvement of the antioxidant property in the reversal of PXR downregulation. Secondly, we have shown that IL-6 and Thaps targeted the same
regulatory sequence (Figs. 2A and 5A) and IL-6 is known to activate the STAT3 signaling pathway (Bode et al., 2012). Thirdly, treatment with Thaps induced the expression of IL-6 (Fig. 5C), suggesting that increased expression of IL-6 at least in part plays a role in Thaps-mediated downregulation of PXR. On the other hand, it remains to be determined whether increased expression of IL-6 by Thaps represents a general phenomenon among ER stressors and diseases associated with ER stress. The connection between Thaps and IL-6, nevertheless, provides a mechanistic understanding of how ER stress conditions may exert differential effect on the expression of PXR depending on the increased secretion of cytokines such as IL-6.

STAT3 is a DNA-sequence-specific transcription factor (Bode et al., 2012). However, the PXR promoter regulatory sequence targeted by Thaps and IL-6 does not harbor a consensus STAT3 element. Instead, this sequence contains 2 adjacent elements that were recognized by HNF4α and C/EBP proteins, respectively. It is therefore assumed that STAT3 decreases the expression of PXR by regulating the expression of HNF4α, C/EBPs, or both. While it is not clear whether STAT3 down-regulates HNF4α, it was reported that STAT3 up-regulated the expression of C/EBPβ (Anastasov et al., 2010). Furthermore, STAT3 was shown to interact directly with C/EBPβ. Given the fact that cotransfection of LAP increased PXR expression (Fig. 2C), the STAT3-C/EBPβ complex likely exerts repressive activity. Alternatively, such complex no longer acts on the PXR promoter, thus functioning as a dominant negative in comparison with LAP. The C/EBP family has several members and they all bind to same or similar DNA elements (Tsukada et al., 2010). It is conceivable that other C/EBP members likely participate in the regulated expression of PXR during ER stress.

One of the interesting findings in this study is the unique interplay between C/EBPβ and HNF4α. In the reporter experiment, disruption of the HNF4α element completely eliminated the repressive activity in response to Thaps (Fig. 2B). In contrast, disruption of the C/EBP element diminished the repression to a much lesser extent (Fig. 2B). These observations suggested that HNF4α played an essential or a greater role than a C/EBP protein (probably C/EBPβ) in supporting the expression of PXR. However, transfection of HNF4α surprisingly caused less increases of PXR mRNA than cotransfection of LAP (an active form of C/EBPβ) (Fig. 2C). One explanation is that LAP functioned as a transactivator of HNF4α and/or LAP enhanced the activity of HNF4α. In support of the last possibility, LAP was shown to increase nuclear translocation of HNF4α (Shen et al., 2000).

C/EBPβ mRNA produces several in-frame translated polypeptides including LAP*, LAP, and LIP. Under normal conditions, LAP is the most abundant form. While LAP and LAP are transactivators, LIP acts as a transcriptional repressor (Tsukada et al., 2010). It is generally accepted that the repressive activity of LIP is achieved by forming non-functional dimmer with C/EBP activating members and/or a DNA-binding dominant negative. It is also accepted that the relative abundance of various C/EBPβ forms (eg, LAP vs LIP) largely depends on the relative efficiency of the initiation codons for translation. Interestingly, Thaps treatment caused an 11-fold increase of C/EBPβ mRNA (Fig. 3A), and yet the increase in proteins was detected on LIP but not LAP or LAP* (Fig. 3A). One explanation is that the initiation codon for
Thaps was more efficient under ER stress condition induced by Thaps. It has been reported that LAP can be converted into LIP through proteolytic digestion through an unknown protease. It is likely that such a protease(s) is the one that decreases the expression of PXR in the presence of Thaps. Nevertheless, EMSA experiment detected increases in DNA binding, apparently by LIP (Fig. 4B). In contrast, the intensity of the shifted bands by LAP and LAP* was slightly decreased in nuclear extracts of cells treated with Thaps. Based on ChIP experiment, however, the increased LIP did not lead to increases in the occupancy of the C/EBP element in the PXR promoter, although the same antibody was used in both EMSA and ChIP experiments. One explanation is that chromatin-bound LIP posed a configuration that hindered the epitope from being recognized by this antibody. Alternatively, LIP normally does not bind to the C/EBP element in the PXR promoter under the native condition (ie, cell), although it did so under non-cellular context (ie, EMSA). Nonetheless, cotransfection of LIP indeed conferred potent repressive activity toward the PXR promoter reporter (data not shown).

In summary, our study presents several important conclusions. Firstly, ER stressors decreased the expression of PXR and the induction of CYP3A4, pointing to the possibility of reduced capacity of drug metabolism and detoxication during ER stress condition. Secondly, the decreased expression of PXR was a sequence-specific event through adjacent HNF4α-C/EBP elements. Cotransfection of HNF4α or LAP restored PXR expression, suggesting that factors, altering the activity of these transcription factors, likely affect the expression of PXR and its target genes. Thirdly, ER stressors and IL-6 targeted the same element in repressing PXR, establishing a novel functional link. This is particularly of significance as such connection suggests that ER stress conditions may vary in suppressing PXR expression depending on the enhanced secretion of cytokines such as IL-6.

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