Alterations of Histone Modifications Contribute to Pregnane X Receptor-mediated Induction of CYP3A4 by Rifampicin

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ABBREVIATIONS:

CYP3A4: cytochrome P450 3A4; H3K4me3: histone 3 lysine 4 trimethylation;
H3K27me3: histone 3 lysine 27 trimethylation; NCOA6: nuclear receptor coactivator 6;
PXR: pregnane X receptor; ASCOM: ASC-2 complex; MLL3: mixed lineage leukemia 3;
MLL4: mixed lineage leukemia 4; PXRE: PXR response element; UTX: lysine demethylase 6A; SRC-1: steroid receptor coactivator 1;
ABSTRACT

Cytochrome P450 3A4 (CYP3A4) is one of the major drug-metabolizing enzymes in human and is responsible for the metabolism of 60% of the clinically used drugs. Many drugs are able to induce the expression of CYP3A4, which usually causes drug-drug interactions and adverse drug reactions. This study aims to explore the role of histone modifications in rifampicin-induced expression of CYP3A4 in LS174T cells. We found that the induction of CYP3A4 mRNA (4- to 15-fold) by rifampicin in LS174T cells was associated with increased levels of histone H3 lysine 4 trimethylation (H3K4me3, above 1.8-fold) and H3 acetylation (above 2-fold) and a decreased level of histone H3 lysine 27 trimethylation (H3K27me3, about 50%) in the CYP3A4 promoter. Rifampicin enhanced the recruitment of nuclear receptor coactivator 6 (NCOA6, above 3-fold) and histone acetyltransferase p300 (p300, above 1.6-fold) to the CYP3A4 promoter. Silencing NCOA6 or p300 by shRNAs resulted in inhibition of the CYP3A4 induction as well as the altered levels of H3K4me3, H3K27me3, or H3 acetylation in the CYP3A4 promoter, respectively. Knockdown of pregnane X receptor (PXR) expression not only suppressed the recruitment of NCOA6 and p300, but also abolished the changes of H3K4me3, H3K27me3, and H3 acetylation levels in the CYP3A4 promoter caused by rifampicin. Moreover, rifampicin treatment enhanced the nuclear accumulation and interactions between PXR and NCOA6/p300. In conclusion, we show that the alterations of histone modifications contribute to the PXR-mediated induction of CYP3A4 by rifampicin.
Introduction

Human cytochrome P450 3A4 (CYP3A4) plays an important role in the metabolism of 60% clinical drugs and is responsible for many drug-drug interactions due to inducibility by numerous inducers (Liu et al., 2007; Novotna and Dvorak, 2014; Raucy, 2003). Drug-drug interactions caused by the induction of CYP3A4 have posed many challenges to drug development and clinical safety (Baciewicz et al., 2013; Cheng et al., 2009; Niemi et al., 2003). Over the past few years, the molecular mechanisms accountable for the regulation of CYP3A4 have been well studied. The induction of CYP3A4 is thought to be based on the activated pregnane X receptor (PXR) (Goodwin et al., 1999), constitutive androstane receptor (CAR) (Moore et al., 2000), or vitamin D receptor (VDR) (Thummel et al., 2001) binding to the CYP3A4 promoter (Schuetz, 2004). However, individual variability in CYP3A4 induction has been observed (Tang et al., 2005). Previously, genetic variations in CYP3A4, its related transporters, and transcriptional factor genes were considered to contribute to the variability (Lamba et al., 2006; Tang et al., 2005; Thirumaran et al., 2012). However, the genetic polymorphisms cannot sufficiently explain the large interindividual variability in the CYP3A4 induction due to their limited frequencies in the general population (Rahmioglu et al., 2011).

Epigenetic modifications, such as histone methylation and acetylation, are important factors in chromatin remodeling and play essential roles in the regulation of gene transcription (Schubeler et al., 2004). The induction of genes by different stimulations, including xenobiotics and endogenous compounds, were found to be associated with histone modifications, such as histone methylation and acetylation (Xie et al., 2015; Xydous et al., 2012; Zhang et al., 2013). Similarly, drug metabolism enzymes and
transporters are also regulated by different epigenetic modifications (Ivanov et al., 2014; Peng and Zhong, 2015). In HepG2 cells, histone H3 acetylation was showed to be associated with trichostatin A-mediated regulation of CYP2E1 (Yang et al., 2010). Transcriptional activation of CYP2C9 was found to be regulated by the epigenetic modification of histone H3 lysine 27 (H3K27) (Englert et al., 2015). Dynamic alterations of histone methylation are associated with ontogenic expression of the Cyp3a genes during mouse liver maturation (Li et al., 2009). Besides, MDR1 gene was proved to be regulated by histone methyltransferase MLL1 in chemoresistance (Huo et al., 2010). More interestingly, histone deacetylase 1 (HDAC1) was showed to be required in carbamazepine-induced CYP3A4 expression in HepG2 cells (Wu et al., 2012). These findings suggest that histone modifications may also be involved in the regulation of CYP3A4 and contribute to its individual variability as well as inducibility.

Epigenetic modifications are often regulated by nuclear receptors via recruitment of cofactors that are necessary for catalyzing distinct types of histone modifications (Green and Han, 2011). Nuclear receptor coactivator 6 (NCOA6, also named ASC-2, PRIP, AIB3, RAP250, NCR, and TRBP) is a transcriptional coactivator of multiple nuclear receptors and belongs to a novel steady-state complex, called ASCOM (for ASC-2 complex), which contains two different histone-modifying enzymes linked to transcriptional activation, histone H3 lysine 4 (H3K4) methyltransferase MLL3/MLL4 and H3K27-demethylase UTX (Lee et al., 2009). Trimethylation of H3K4 (H3K4me3) is an active epigenetic mark for gene transcription, but trimethylation of H3K27 (H3K27me3) is a gene silencing mark. It has been demonstrated that NCOA6 plays an essential role in ligand-dependent regulation of target gene expression by transcriptional factors.
through recruitment of MLL3/MLL4 to promote H3K4 methylation (Ananthanarayanan et al., 2011; Lee et al., 2008). The demethylation of H3K27me3 regulated by UTX was also reported to be required for transcriptional activation (Zha et al., 2015). Moreover, PXR and HNF4α (hepatic nuclear factor 4α) were found to interact with NCOA6 in vitro (Surapureddi et al., 2011). Apart from that, histone acetylation catalyzed by p300, an essential coactivator recruited by transcriptional factors, is reported to be involved in gene induction (Zhang et al., 2013).

In the present study, we investigated whether NCOA6 and p300 as well as histone methylation and acetylation were involved in PXR-mediated induction of CYP3A4 by rifampicin in LS174T cells. LS174T is a human intestinal cell line derived from a 58 year old female with adenocarcinoma of the colon and has been used in studies of the PXR-mediated induction of CYP3A4 by rifampicin (Liu et al., 2012; Tan et al., 2016). We found that NCOA6 and p300 were recruited to the CYP3A4 promoter by PXR after rifampicin treatment and were responsible for the changes of H3K4me3, H3K27me3, and H3 acetylation in the CYP3A4 promoter, further contributing to the induction of CYP3A4.
Materials and Methods

Reagents. Rifampicin was purchased from Sigma-Aldrich (St. Louis, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (BI, Kibbutz Beit Haemek, Israel). Protein A and G magnetic beads were purchased from Life Technologies (Carlsbad, USA). Proteinase inhibitor cocktail was purchased from Roche (Basel, Switzerland). Penicillin, streptomycin, puromycin, ampicillin, dimethylsulfoxide (DMSO), and all other chemicals were purchased from Solarbio Company (Beijing, China), unless otherwise noted.

Plasmids. PSG5-hPXR, a human PXR expression vector, was provided generously by Dr. Wen Xie (Center of Pharmacogenomics, University of Pittsburgh, Pittsburgh, PA, USA). Human NCOA6 expression vector, pCMV2-hNCOA6:V5, was a gift from Kenneth Irvine (Addgene plasmid # 63585) (Oh et al., 2014). Human p300 expression vector, pcDNA3.1-p300, was a gift from Warner Greene (Addgene plasmid # 23252) (Chen et al., 2002). shRNA plasmids that targeting NCOA6 (HSH005707-LVRU6P) or p300 (HSH004897-LVRU6P) were provided by GeneCopoeia (Rockville, MD, USA), and shRNA plasmid targeting PXR (sc-44057-SH) was provided by Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA).

Cell culture and treatment. Human colon cancer LS174T cells (obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone) supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin under 5% CO2 at 37°C. For induction study, LS174T cells were treated with rifampicin (10 µM) or solvent (DMSO, 0.1% v/v) for 48, 72, or 96 hrs before mRNA and protein determination.
Transfection and RNA interference. All transfections were performed using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA), following the manufacturer’s instructions. To generate stable cell lines with continuous suppression of specific gene expression, LS174T cells were transiently transfected with a shRNA plasmid that targets NCOA6 (shNCOA6) or p300 (shp300), respectively. After 48 hrs of transfection, a puromycin selection with a concentration of 2 μg/ml was performed for 4 to 6 weeks until small colonies occurred. Then, the colonies were tested for mRNA determination and positive colonies were selected to generate stable cell lines using puromycin selection. Knockdown of PXR expression was performed by transient transfection with a shRNA plasmid (shPXR). For overexpression studies, PSG5-PXR, pCMV2-hNCOA6:V5, and pcDNA3.1-p300 plasmids were transfected into LS174T cells, respectively or together.

RNA isolation and gene expression analysis. RNA was extracted using Trizol reagent (Roche, Basel, Switzerland) and 1 μg of total RNA was reversely transcribed to cDNA using a PrimeScript RT Reagent Kit (Takara Bio, Dalian, China). Relative mRNA expression of genes was determined by real-time quantitative PCR (qPCR) and normalized to GAPDH mRNA using a SYBR green method (Life Technologies, Carlsbad, CA, USA). Primers are shown in Supplemental Table 1.

Western Blot analysis. Total proteins of treated LS174T cells were prepared using RIPA buffer and protein concentrations were determined by a bicinchoninic acid method according to the manufacturer’s instructions (Beyotime Institute of Biotechnology, Hangzhou, China). Total proteins were separated on SDS-PAGE gels (6~12%) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were
incubated with a primary antibody: anti-CYP3A4 (Abcam, Cambridge, MA, USA), anti-PXR (Abcam), anti-NCOA6 (Proteintech, Wuhan, China), anti-p300 (Abcam), or anti-GAPDH after blocked with 5% non-fat milk and were visualized with enhanced chemiluminescence followed by densitometry analysis of blot bands using FluorChem™ E system (Bio-Techne, CA, USA). The relative fold changes of CYP3A4, PXR, NCOA6, and p300 proteins were normalized to GAPDH.

**Chromatin immunoprecipitation (ChIP) analysis.** ChIP was performed on treated cells to determine enriched levels of H3K4me3 (17-678, Millipore, Billerica, MA, USA), H3K27me3 (17-622, Millipore), and H3 acetylation (06-599, Millipore) as well as binding levels of PXR, NCOA6, and p300 in the promoter region of CYP3A4 gene. After treatment, cells were cross-linked with 1% (v/v) formaldehyde for 10 min, then stopped by glycine (125 mM) for 5 min at room temperature. Cells were washed twice with cold PBS, harvested and were lysed in SDS lysis buffer (0.1% SDS, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% Triton-X) with a proteinase inhibitor cocktail on ice for 15 min. Cell lysates were sonicated with a Bioruptor Pico sonication system (Diagenode, Belgium) to achieve DNA fragments between 200~1,000 bp and centrifuged at 12,000 g for 10 min at 4°C. 10% of the supernatants were taken as chromatin input and aliquot of supernatants were incubated with antibodies overnight at 4°C after diluted with ChIP dilution buffer (1 mM EDTA, 50 mM Tris-HCl, pH 8.0). A mouse IgG (Millipore, Billerica, MA, USA) was used as a negative control. Then, prewashed Dynabeads® Protein A and G were added to each sample and incubated for 2 hrs and washed three times with a high salt wash buffer (0.1% SDS, 1% Triton X-100, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl), three times with a low salt wash buffer (0.1% SDS, 1% Triton...
X-100, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl), once with a LiCl buffer (0.25 M LiCl, 1% NP40, and 1% sodium deoxycholate), twice with a TE buffer (1 mM EDTA and 20 mM Tris-HCl, pH 8.0). After all washes, samples were eluted with an elution buffer (1% SDS, 0.5 M EDTA, and 1 M Tris-HCl, pH 8.0) and incubated with 200 mM NaCl at 65°C for overnight to reverse cross-linking. Then samples were incubated with Proteinase K for 1.5 hrs at 55°C and DNA was purified using a PCR purification kit (QIAGEN, Germany) followed by real-time quantitative PCR analysis with the primers (Supplemental Table 1) using a SYBR green method, enrichment was calculated as percentage of chromatin input.

**Immunofluorescence.** LS174T cells that grown on coverslip-coated 24-well plate and treated with rifampicin or solvent for 48 hrs were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, then blocked with 1% BSA for 1.5 hrs followed by overnight incubation of a primary antibody at 4°C and 1.5 hrs incubation of a secondary antibody at room temperature: for PXR localization, an anti-PXR (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a Alexa Fluor 647-conjugated rabbit secondary antibody (1:200, Abcam, Cambridge, MA, USA) were used, for NCOA6 or p300 localization, an anti-NCOA6 (1:50, Proteintech, Wuhan, China) or anti-p300 (1:100, Abcam, Cambridge, MA, USA) with a Alexa Fluor 488-conjugated mouse secondary antibody (1:200, Abcam, Cambridge, MA, USA) were used. DAPI (Beyotime Institute of Biotechnology, Hangzhou, China) was used to stain nuclei. Fluorescence was observed using a laser confocal microscope FluoView FV1000 (OLYMPUS, Japan).

**Co-immunoprecipitation (Co-IP).** LS174T cells were transiently cotransfected with PSG5-hPXR and pCMV2-hNCOA6:V5 or pcDNA3.1-p300 plasmids, 24 hrs after
transfection cells were treated with rifampicin or solvent for additional 48 hrs and were harvested to perform Co-IP using a Pierce Classic IP Kit (Thermo Scientific, USA) according to the manufacturer's instructions with a few modifications. Briefly, whole cell lysates were diluted with 1 × TBS and immunoprecipitated with an anti-NCOA6 or anti-p300 antibody, a rabbit anti-IgG as a negative control. The immunoprecipitates were captured with Dynabeads® Protein A/G followed by washes and then eluted for Western Blot analysis.

**Statistical Analysis.** Data are expressed as mean ± SD. Statistical significance between groups was analyzed by Student t-test, or one-way ANOVA with Bonferroni’s post hoc test or Dunnett’s test using SPSS for Windows, version 17.0 (IBM, NY, USA). A value of $P<0.05$ was considered to be statistically significant.
Results

Rifampicin-induced CYP3A4 expression was accompanied with altered levels of H3K4me3, H3K27me3, and H3 acetylation in the CYP3A4 promoter. The induction of CYP3A4 by rifampicin (10 μM) in LS174T cells was assessed by real-time quantitative PCR and Western Blot. Rifampicin treatment for 48 hrs, 72 hrs, or 96 hrs induced the expression of CYP3A4 mRNA by 4.5-fold (95% CI: 1.76 to 4.91), 5.9-fold (95% CI: 3.20 to 6.34), and 15.0-fold (95% CI: 12.25 to 15.40), respectively (Fig. 1A), and protein by 1.6-fold (95% CI: 0.22 to 0.91), 1.8-fold (95% CI: 0.49 to 1.79), and 2.9-fold (95% CI: 1.56 to 2.24), respectively (Fig. 1B), compared to a solvent control.

To reveal the influence of rifampicin on histone modifications, the enriched levels of H3K4me3, H3 acetylation, and H3K27me3 around the PXR response elements (PXRE) in the promoter of CYP3A4 were determined by ChIP analysis in rifampicin-treated LS174T cells. Since the induction of CYP3A4 by rifampicin is based on activation of PXR, a set of ChIP-qPCR primers were designed to cover the distal (-7,898 ~-7,733 bp) and proximal (-172~149 bp) PXRE in the promoter of CYP3A4 gene, accompanied with a pair of primers (5,397~5,500 bp) located in intron 2 of CYP3A4 gene as a negative control (Fig. 2A). After rifampicin treatment for 48 hrs or 96 hrs, the levels of H3K4me3 were increased by 1.8-fold in distal PXRE region (95% CI: 0.30 to 1.16 and 95% CI: 0.16 to 1.02, respectively), and higher increase (2.6-fold, 95% CI: 0.12 to 3.44 and 3.5-fold, 95% CI: 1.47 to 4.80, respectively) was observed in the proximal PXRE region (Fig. 2B, 2C, and Supplemental Fig. 1A). A high level of H3K27me3 in promoter region of a gene is a mark, which is associated with gene silence. In this study, rifampicin treatment (48 hrs and 96 hrs) was found to decrease the levels of H3K27me3 in both distal (60%,
95% CI: -0.91 to -0.34 and 45%, 95% CI: -0.71 to -0.13, respectively) and proximal (30%, 95% CI: -0.49 to -0.17 and 53%, 95% CI: -0.68 to -0.36, respectively) PXRE regions (Fig. 2E, 2F, and Supplemental Fig. 1B). Moreover, H3 acetylation, which is often observed in promoter of an active gene, was also increased by rifampicin in the same regions (Fig. 2H, 2I, and Supplemental Fig. 1C). These results suggest that alterations of histone methylation and acetylation are involved in rifampicin-induced CYP3A4 expression in LS174T cells.

**Rifampicin increased NCOA6 and p300 binding to PXRE in the CYP3A4 promoter.** Since NCOA6 and p300 have been reported to be associated with H3K4me3, H3K27me3, and H3 acetylation levels, respectively, the binding of NCOA6 and p300 to the PXRE regions in the CYP3A4 promoter was analyzed by ChIP analysis. PXR binding was also analyzed as the induction of CYP3A4 by rifampicin is based on its occupancy. The enrichments of PXR at both distal and proximal PXRE regions were increased by 1.7-fold (95% CI: 0.29 to 1.10) and 2.1-fold (95% CI: 0.50 to 2.33) after rifampicin treatment for 48 hrs, and increased by 3.3-fold (95% CI: 1.90 to 2.72) and 3.8-fold (95% CI: 1.68 to 3.97) after treatment for 96 hrs, respectively (Fig. 3A, 3B, and Supplemental Fig. 1D). The enrichments of NCOA6 at both regions were increased by 3.1-fold (95% CI: 0.10 to 4.09) and 3.2-fold (95% CI: 1.01 to 3.98) after rifampicin treatment for 48 hrs, and were increased by 4.9-fold (95% CI: 1.98 to 5.97) and 6.4-fold (95% CI: 4.31 to 6.58) after 96 hrs of the treatment (Fig. 3D, 3E, and Supplemental Fig 1E). The enrichments of p300 were increased by 2.2-fold (95% CI: 0.24 to 2.23) and 1.6-fold (95% CI: 0.12 to 0.98) after rifampicin treatment for 48 hrs and increased by 3.7-fold (95% CI: 1.72 to 3.70) and 3.4-fold (95% CI: 1.93 to 2.79) after 96 hrs of the
treatment (Fig. 3G, 3H, and Supplemental Fig. 1F). These findings indicate that recruitment of NCOA6 and p300 is involved in PXR-mediated induction of CYP3A4 by rifampicin and can be associated with the changes of H3K4me3, H3K27me3, and H3 acetylation levels in the CYP3A4 promoter.

**NCOA6 and p300 are the essential factors in PXR-meditated induction of CYP3A4 by rifampicin.** To explore the specific roles of NCOA6 and p300 in the CYP3A4 induction, stable cell lines with decreased expression of NCOA6 or p300 were generated as mentioned in the methods. Transient transfection was performed to knockdown the expression of PXR in LS174T cells to confirm its function in the CYP3A4 induction. The levels of mRNA and protein expression of PXR were decreased after shRNA transfection compared with a negative control group (Fig. 4A and 4B), the basal expression of CYP3A4 mRNA was decreased by 31% (95% CI: 0.08 to 0.53) (Fig. 4C). To study the effect of silencing PXR on rifampicin-induced CYP3A4 expression, shRNA transfected cells were treated with rifampicin and CYP3A4 expression was determined. As a result, silencing the expression of PXR abolished the induction of CYP3A4 (Fig. 4C). Moreover, similar results were observed in stably transfected LS174T cells that possessed a lower expression level of NCOA6 or p300. Silencing the expression of NCOA6 not only reduced 64% (95% CI: 0.33 to 0.94) of the basal expression of CYP3A4, but also totally abolished the induced expression of CYP3A4 (Fig. 4F). Besides, silencing of p300 also reduced the basal and rifampicin-induced expression of CYP3A4 (decreased by 30% [95% CI: 0.07 to 0.54] and 70% [95% CI: 2.72 to 3.18], respectively) (Fig. 4I). All together, these results show that NCOA6 and p300 are indispensable in PXR-mediated induction of CYP3A4 by rifampicin.
NCOA6 and p300 are required in rifampicin-induced changes of histone modifications in the CYP3A4 induction. To further confirm the effects of NCOA6 and p300 on histone modifications in the induction of CYP3A4 by rifampicin, ChIP analysis was performed after silencing the expression of NCOA6 and p300, respectively (Fig. 5 and Supplemental Fig. 2). Firstly, knockdown of NCOA6 expression was performed by stable transfection to study the effect of silencing NCOA6 expression on rifampicin-induced alterations of H3K4me3 and H3k27me3 levels. Silencing of NCOA6 decreased the basal level of H3K4me3 and increased the basal level of H3K27me3 in the CYP3A4 promoter compared with a transfection control group (Fig. 5A and 5B). The changes of H3K4me3 and H3K27me3 in the CYP3A4 promoter by rifampicin were also abolished after silencing of NCOA6: the levels of H3K4me3 in rifampicin-treated shNCOA6 group were decreased by 57% (95% CI: 0.78 to 1.44) in the distal PXRE region and 83% (95% CI: 5.16 to 6.58) in the proximal PXRE region compared with the rifampicin-treated control group (Fig. 5A, 5B, and Supplemental Fig. 2A); the levels of H3K27me3 were increased by 5.3-fold (95% CI: -0.69 to -0.34) and 14.0-fold (95% CI: -0.94 to -0.54), respectively (Fig. 5D, 5E, and Supplemental Fig. 2B). Besides, a decrease of H3 acetylation in the CYP3A4 promoter was observed after knockdown of p300 expression, the increase of H3 acetylation by rifampicin was reduced by 80% (95% CI: 5.94 to 8.01) and 76% (95% CI: 4.18 to 5.41) in the distal and proximal PXRE regions, respectively, when compared with the control transfection group treated with rifampicin (Fig. 5G, 5H, and Supplemental Fig. 2C). These findings demonstrate that NCOA6 and p300 are responsible for the alterations of H3K4me3, H3K27me3, and H3 acetylation in the CYP3A4 promoter with or without rifampicin.
PXR recruits NCOA6 and p300 to the CYP3A4 promoter and modulates the epigenetic changes of H3K4me3, H3K27me3, and H3 acetylation. Then, the effects of silencing PXR expression on levels of H3K4me3, H3K27me3, and H3 acetylation as well as the enrichment of NCOA6 and p300 in the CYP3A4 promoter were determined (Fig. 6 and Supplemental Fig. 2). Knockdown of PXR expression reduced the levels of H3K4me3 and H3 acetylation and increased the level of H3K27me3 in the CYP3A4 promoter regardless of rifampicin treatment. The levels of H3K4me3 in the distal and proximal PXRE regions were reduced by 68% (95% CI: 0.81 to 1.74) and 90% (95% CI: 6.48 to 10.90) in rifampicin-treated PXR silencing group when compared with rifampicin-treated control group (Fig. 6A, 6B, and Supplemental Fig. 2D); whereas, the levels of H3K27me3 were increased by 8.9-fold (95% CI: -1.11 to -0.44) and 6.6-fold (95% CI: -2.25 to -1.30) (Fig. 6D, 6E, and Supplemental Fig. 2E); the H3 acetylation levels were reduced by 52% (95% CI: 1.07 to 1.86) and 80% (95% CI: 1.15 to 1.77) in rifampicin-treated PXR silencing group (Fig. 6G, 6H, and Supplemental Fig. 2F). Moreover, silencing the expression of PXR also decreased the recruitment of NCOA6 and p300 to the CYP3A4 promoter in both rifampicin-treated and untreated cells, the enrichment of NCOA6 was reduced by at least 80% while p300 was reduced by 70% in rifampicin-treated PXR silencing group (Fig. 6J-N, and Supplemental Fig. 2G and 2H). These results suggest that PXR is essential for the enrichment of NCOA6 and p300 and the epigenetic changes of histone modifications in the CYP3A4 promoter.

Next, we performed an immunofluorescence staining experiment to determine the effect of rifampicin on nuclear accumulation of PXR, NCOA6, and p300. Rifampicin increased the nuclear accumulation of PXR, NCOA6, and p300 (Fig. 7A, column 5).
Besides, the co-localization of PXR and NCOA6/p300 was increased in rifampicin treated cells (Fig. 7A, column 5, rows II and IV). Moreover, the Co-IP results showed that NCOA6 and p300 directly interacted with PXR and the interaction was enhanced in rifampicin treated LS174T cells [Fig. 7B and 7C]. These results suggest that rifampicin increases the nuclear accumulation of PXR, NCOA6, and p300, and enhances the interactions between PXR and NCOA6/p300.
Discussion

Induction of drug-metabolizing enzymes and transporters is an important cause of drug-drug interactions, especially for the induction of CYP3A4, the most abundant isoform of cytochrome P450. PXR is the most important nuclear receptor that is responsible for the induction of CYP3A4 by a broad range of xenobiotics. Increasing evidence indicates that histone modifications are important to nuclear receptor-mediated transcription of target genes (Huo et al., 2010; Xie et al., 2009). In this study, we found that levels of H3K4me3 and H3 acetylation in the CYP3A4 promoter, two marks for activation of gene transcription, were elevated by rifampicin treatment in LS174T cells, while the level of H3K27me3 (a mark for gene silencing) was decreased. Then, we found that NCOA6 and p300 were required in the PXR-mediated induction of CYP3A4 and were associated with histone methylation and acetylation in the CYP3A4 promoter.

Epigenetic modifications like histone methylation, acetylation, phosphorylation, and ubiquitination have been shown to play important roles in the regulation of gene expression. Drug-metabolizing enzymes were also reported to be regulated by histone modifications. For instance, CYP2C9 was found to be regulated by MED25 through the epigenetic modification of H3K27. MED25 was recruited to the CYP2C9 promoter by HNF4α and promoted H3K27 acetylation while repressed its methylation (Zha et al., 2015). In the current study, we determined the status of H3K4me3, H3K27me3, and H3 acetylation in the CYP3A4 promoter upon rifampicin exposure as these histone modifications are commonly seen in the regulation of gene expression. We found that rifampicin elevated the levels of H3K4me3 and H3 acetylation while attenuated the
levels of H3K27me3. These results indicate that histone methylation and acetylation are involved in the induction of CYP3A4 by rifampicin.

However, epigenetic modifications occur as executors rather than initiators in the regulation of gene expression. Alterations of environment, physiology, and pathology are considered to be the causes of epigenetic changes (Peng and Zhong, 2015). Nuclear receptors are suggested to be the sensors that respond to extracellular changes, and they have been identified to interact with epigenetic modification enzymes after activated by ligands to control gene expression (Green and Han, 2011). We investigated the mechanism under the changes of histone methylation and acetylation in the CYP3A4 promoter caused by rifampicin. The enrichment of NCOA6 and p300 at the PXRE regions in the CYP3A4 promoter was significantly increased after rifampicin treatment when compared to a control group and the enrichment was consistent with changes of H3K4me3, H3 acetylation, and H3K27me3. Among the cofactors that related with nuclear receptors, NCOA6 has been demonstrated to be critical for the trimethylation of H3K4 in gene transcription as it belongs to a part of ASCOM, which contains H3K4 methyltransferase MLL3/4 (Ananthanarayanan et al., 2011). We found that silencing NCOA6 resulted in a remarkable decrease of H3K4me3, but a considerable increase of H3K27me3 in the CYP3A4 promoter. Apart from H3K4 methyltransferase MLL3/4, the ASCOM also contains H3K27 demethylase UTX. Thus, the involvement of ASCOM in transcriptional activation was carried out by altering the trimethylation of H3K4 and H3K27 (He et al., 2015). Besides, the histone acetyltransferase p300 plays an important role in nuclear receptor-mediate gene expression through elevating levels of histone H3 and H4 acetylation near nuclear
receptor binding sites in promoter of a target gene (Green and Han, 2011; Tsai and Fondell, 2004). Indeed, increase of p300 enrichment near the PXR binding sites in the \textit{CYP3A4} promoter was observed after LS174T cells were incubated with rifampicin. And the increase was correlated with the induction of CYP3A4 and increase of H3 acetylation by rifampicin as they were both diminished after the silence of p300 expression. More importantly, we found that the recruitment of NCOA6 and p300 to the \textit{CYP3A4} promoter were mediated by PXR. Knockdown of the expression of PXR resulted in significant inversion of alteration of histone modifications and enrichment of NCOA6 and p300 in the \textit{CYP3A4} promoter, which was consistent with the decrease of CYP3A4 induction. It has been reported that NCOA6 interacts with PXR through the first LXXLL motif (Surapureddi et al., 2011). In the immunofluorescence and Co-IP experiments, we confirmed the interaction between NCOA6/p300 and PXR and found that rifampicin enhanced the interaction. Thus, these results indicate that PXR recruits NCOA6 and p300 to the \textit{CYP3A4} promoter region and modulates histone methylation and acetylation to facilitate the CYP3A4 induction.

In addition to NCOA6 and p300, some of the coactivators that interact with PXR could affect chromatin remodeling or histone modification patterns, such as steroid receptor coactivator 1 (SRC-1). It has been reported that SRC-1 together with p300 plays crucial roles in dioxin-induced expression of CYP1A1 and CYP1B1 in MCF-7 cells (Taylor et al., 2009). SRC-1 has been reported to interact with PXR and regulate CYP3A4 induction (Li and Chiang, 2006). However, its effect on histone modifications, like H3 acetylation in the \textit{CYP3A4} promoter remains unclear. Further studies focusing on SRC-1 and its role in histone modification patterns in the \textit{CYP3A4} promoter induced
by PXR ligands will provide valuable findings in understanding the epigenetic regulation of CYP3A4 induction.

The importance of epigenetic regulation of drug metabolism-related genes has been reviewed recently (Ivanov et al., 2014; Peng and Zhong, 2015; Tang and Chen, 2015). Epigenetic regulation of gene expression is modulated by environmental factors, such as drugs, diets, and environmental pollutants (Choudhuri et al., 2010). For example, it has been demonstrated that dynamic H3K27 acetylation is associated with VEGFA-stimulated changes in chromatin conformation and transcriptional responses (Zhang et al., 2013). Apparently, epigenetic changes in drug-metabolizing enzymes caused by environmental factors could be a reason for the interindividual differences in drug responses and drug-drug interactions. In addition to CYP3A4, PXR regulates a variety of other P450 enzymes, phase II drug metabolizing enzymes, and drug transporters in response to xenobiotics (Chai et al., 2013). Further researches using primary hepatocytes and transgenic mice in the determination of epigenetic regulation of drug-metabolizing enzymes and transporters mediated by PXR will be performed to provide new insights in the variations of drug responses and will be beneficial to precision medicine in future.

In conclusion, this study reveals that alterations of histone methylation and acetylation are involved in the CYP3A4 induction by rifampicin through the activation of PXR and recruiting histone methyltransferase related factor NCOA6 and histone acetyltransferase p300.
Acknowledgements

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Authorship Contributions:

*Participated in research design:* Yan, Zhong, Kan, and Zhang.

*Conducted experiments:* Yan, Wang, Liu, and Nie.

*Performed data analysis:* Yan, Wang, Zhang.

*Wrote or contributed to the writing of the manuscript:* Yan, Zhong, Zhang
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Footnotes:

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Figure legends

**Fig. 1.** Effect of rifampicin on the expression of CYP3A4 mRNA and protein in LS174T cells. LS174T cells were seeded onto 24 well plate and treated with a solvent DMSO control (0.1%, v/v) or rifampicin (RIF) (10 μM) for 48, 72, and 96 hrs, respectively. Then cells were harvested for determination of mRNA by real-time quantitative PCR (qPCR) (A) and protein by Western Blot (B). Data are shown as mean ± SD of three independent experiments. *p<0.05, **p<0.01 versus control (one-way ANOVA followed by Dunnett’s test).

**Fig. 2.** Effect of rifampicin on the levels of histone modifications around the PXR response elements (PXRE) in the CYP3A4 promoter. LS174T cells were treated with a solvent DMSO control (0.1%, v/v) or rifampicin (10 μM) for 48 or 96 hrs, followed by chromatin immunoprecipitation (ChIP) analysis. Enrichment was calculated as percentage of chromatin input. (A) qPCR primers that cover different sites near the PXRE in the CYP3A4 promoter, a pair of primers locate in intron 2 was performed as a negative control. The relative levels of H3K4me3 (B-D), H3K27me3 (E-G), and H3 acetylation (H-J) in the CYP3A4 promoter region and were determined. Data are shown as mean ± SD of three independent experiments. *p<0.05, **p<0.01 versus control (one-way ANOVA followed by Dunnett’s test).

**Fig. 3.** Effect of rifampicin on the enrichment of NCOA6 and p300 around the PXRE in the CYP3A4 promoter. LS174T cells were treated with a solvent control (0.1% DMSO,
v/v) or rifampicin (10 μM) for 48 or 96 hrs, followed by ChIP analysis. (A-C) Enrichment of nuclear receptor PXR, (D-F) cofactor NCOA6, and (G-I) histone acetyltransferase p300 in the CYP3A4 promoter after LS174T cells were treated with the solvent control or rifampicin. Data are shown as mean ± SD of three independent experiments. *p<0.05, **p<0.01 versus control (one-way ANOVA followed by Dunnett’s test).

**Fig. 4.** Functions of PXR, NCOA6, and p300 in the induction of CYP3A4 by rifampicin. Knockdown of target genes by RNA interference (RNAi) in LS174T cells was performed by transfection of shRNA plasmids. Transiently or stably transfected cells were treated with a solvent DMSO control (0.1%, v/v) or rifampicin (10 μM) for 48 hrs before RNA isolation. Knockdown of PXR by RNAi on mRNA (A) and protein levels (B). Effect of silencing PXR expression on CYP3A4 mRNA and the induction by rifampicin (C). Knockdown of NCOA6 by RNAi on mRNA (D) and protein levels (E). Effect of silencing NCOA6 expression on CYP3A4 mRNA and induction by rifampicin (F). Knockdown of p300 by RNAi on mRNA (G) and protein levels (H). Effect of silencing p300 expression on CYP3A4 mRNA and induction by rifampicin (I). Data are shown as mean ± SD of three independent experiments. *p<0.05, **p<0.01 versus control (student’s t test or one-way ANOVA followed by Bonferroni’s post hoc test).

**Fig. 5.** Effects of silencing NCOA6 or p300 expression on alteration of histone methylation or acetylation levels of the CYP3A4 promoter. Knockdown of NCOA6 or p300 was performed by transfecting shRNA plasmids into LS174T cells followed by treatment with a solvent DMSO control (0.1%, v/v) or rifampicin (10 μM) for 48 hrs, and
cells were harvested for ChIP analysis. The relative levels of H3K4me3 (A-C) or H3K27me3 (D-F) in the CYP3A4 promoter in the control and treated groups. The relative levels of H3 acetylation (G-I) in the CYP3A4 promoter in control and treated groups. Data are shown as mean ± SD of three independent experiments. *p<0.05, **p<0.01 (one-way ANOVA followed by Bonferroni’s post hoc test).

Fig. 6. Effects of silencing PXR expression on alterations of histone methylation and acetylation levels as well as enrichment of NCOA6 and p300 in the CYP3A4 promoter. Knockdown of PXR was performed by transiently transfecting shRNA plasmids into LS174T cells followed by treatment with a solvent DMSO control (0.1%, v/v) or rifampicin (10 μM) for 48 hrs and cells were harvested for ChIP analysis. The relative levels of H3K4me3 (A-C), H3K27me3 (D-F), H3 acetylation (G-I) in the CYP3A4 promoter in control and treated groups. The relative enrichment of NCOA6 (J-L) and p300 (M-O) in the CYP3A4 promoter in control and treated groups. Data are shown as mean ± SD of three independent experiments. *p<0.05, **p<0.01 (one-way ANOVA followed by Bonferroni’s post hoc test).

Fig. 7. Effects of rifampicin on the nuclear accumulation of PXR, NCOA6, and p300 as well as interactions between PXR and NCOA6/p300. (A) Representative immunofluorescence images of PXR, NCOA6, and p300 in the treated LS174T cells. Bar = 10 μm. All cells were stained with DAPI (blue, column 1, row I, II, III, and IV) to visualize the nuclei. Cells were stained with an antibody of anti-NCOA6 (green, column 2, rows I and II), anti-p300 (green, column 2, rows III and IV), and anti-PXR (red,
column 3, rows I, II, III, and IV) to visualize the nuclear and cytoplasmic distribution of NCOA6, p300, and PXR. (B) LS174T cells were cotransfected with PXR and NCOA6 or p300 expression plasmids, and further incubated with rifampicin (10 μM) or a solvent DMSO control (0.1%, v/v) for 48 hrs after the transfection. Cells were harvested for co-immunoprecipitation and detected by Western Blot. All experiments were performed for three independent replicates.
Figure 1

A

B

CYP3A4

GAPDH

relative fold changes of CYP3A4 mRNA

relative fold changes of CYP3A4 protein

DMSO control

RIF 48 hrs

RIF 72 hrs

RIF 96 hrs
Figure 2

A

ChIP-qPCR primer locations in CYP3A4 gene

Primer 1: -8015~7793
Primer 2: -7881~7760
Primer 3: -7805~7682
Primer 4: -454~308
Primer 5: -341~218
Primer 6: -263~108
Primer 7: 5397~5500

B

HBK4me3 (-7881~7760 bp)

C

HBK4me3 (-263~108 bp)

D

HBK4me3 (5397~5500 bp)

E

H3K27me3 (-7881~7760 bp)

F

H3K27me3 (-263~108 bp)

G

H3K27me3 (5397~5500 bp)

H

H3 acetylation (-7881~7760 bp)

I

H3 acetylation (-263~108 bp)

J

H3 acetylation (5397~5500 bp)
Figure 3

A. PXR (-7881~7760 bp)

B. PXR (-263~108 bp)

C. PXR (5397~5500 bp)

D. NCOA6 (-7881~7760 bp)

E. NCOA6 (-263~108 bp)

F. NCOA6 (5397~5500 bp)

G. p300 (-7881~7760 bp)

H. p300 (-263~108 bp)

I. p300 (5397~5500 bp)
Figure 4

A. Relative fold changes of PXR mRNA

B. Western blot of PXR and GAPDH

C. Relative fold changes of CYP2A4 mRNA

D. Relative fold changes of NCOA6 mRNA

E. Western blot of NCOA6 and GAPDH

F. Relative fold changes of CYP2A4 mRNA

G. Relative fold changes of p300 mRNA

H. Western blot of p300 and GAPDH

I. Relative fold changes of CYP2A4 mRNA
Alterations of Histone Modifications Contribute to Pregnane X Receptor-mediated Induction of CYP3A4 by Rifampicin

Liang Yan, Yiting Wang, Jingyang Liu, Yali Nie, Xiao-bo Zhong, Quancheng Kan, and Lirong Zhang
Supplemental Figure 1. Effect of rifampicin on the levels of histone modifications and enrichment of PXR, NCOA6, p300 near distal (-8,015~-7,793 bp and -7,805~-7,682 bp) and proximal (-454~-308 bp and -341~-218 bp) PXR response element (PXRE) in CYP3A4 promoter. LS174T cells were treated with solvent DMSO control (0.1%, v/v) or rifampicin (10 μM) for 48 or 96 hrs, followed by chromatin immunoprecipitation (ChIP) analysis, enrichment was calculated as percentage of chromatin input. The relative levels of H3K4me3 (A), H3K27me3 (B), and H3 acetylation (C) as well as PXR (D), NCOA6 (E), p300 (F) in the CYP3A4 promoter region and were determined. Data are shown as mean ± SD of three independent experiments. *p<0.05, **p<0.01 versus control (one-way ANOVA followed by Dunnett’s test).

Supplemental Figure 2. Effect of silencing NCOA6, p300, or PXR expression on alteration of histone modifications or NCOA6, p300 binding near distal (-8,015~-7,793 bp and -7,805~-7,682 bp) and proximal (-454~-308 bp and -341~-218 bp) PXRE in CYP3A4 promoter. Knockdown of NCOA6, p300 or PXR was performed by transfecting shRNA plasmids into LS174T cells followed by treatment with a solvent DMSO control (0.1%, v/v) or rifampicin (10 μM) for 48 hrs, and cells were harvested for ChIP analysis, enrichment was calculated as percentage of chromatin input. The effect of silencing NCOA6 on the levels of H3K4me3 (A), H3K27me3 (B), the effect of silencing p300 on the levels of H3 acetylation (C), the effect of silencing PXR on the levels of H3K4me3 (D), H3K27me3 (E), H3 acetylation (F) and NCOA6 (G), p300 (H) in CYP3A4 promoter compared with control group. Data are shown as mean ± SD of three independent
experiments. *$p<0.05$, **$p<0.01$ (one-way ANOVA followed by Bonferroni’s post hoc test).

**Supplemental Table 1. Real-time quantitative PCR primers for mRNA and chromatin immunoprecipitation analysis**

<table>
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<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
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<td><strong>For mRNA analysis</strong></td>
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<tr>
<td>CYP3A4 forward</td>
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<tr>
<td>reverse</td>
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<td>CAACCTACATGGTCAAGGCATC</td>
<td>164 bp</td>
</tr>
<tr>
<td>reverse</td>
<td>ACACCTCGAGGGTTCCAGTCT</td>
<td></td>
</tr>
<tr>
<td>NCOA6 forward</td>
<td>CAGATTGAAGGGAGGTGCT</td>
<td>122 bp</td>
</tr>
<tr>
<td>reverse</td>
<td>GCCATAGGAAATCCGCCT</td>
<td></td>
</tr>
<tr>
<td>p300 forward</td>
<td>TGCCAAACCAGATGATGCCT</td>
<td>161 bp</td>
</tr>
<tr>
<td>reverse</td>
<td>ATAGCCCCATAGGGCGGTGA</td>
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</tr>
<tr>
<td>GAPDH forward</td>
<td>GCACCGGTCAAGGGCTGAGAC</td>
<td>138 bp</td>
</tr>
<tr>
<td>reverse</td>
<td>TGGTGAAGACGCAGTGGGA</td>
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</tr>
<tr>
<td><strong>For ChIP analysis</strong></td>
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<td></td>
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<tr>
<td>Primer 1: -8,015~7,793 bp</td>
<td>GAAAGTCCTGGTTGGCG</td>
<td>222 bp</td>
</tr>
<tr>
<td>forward</td>
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</tr>
<tr>
<td>reverse</td>
<td>TGGAGAAAGCGAGGCT</td>
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</tr>
<tr>
<td>Primer 2: -7,881~7,760 bp</td>
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<td>121 bp</td>
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MOL #108225

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 3: -7,805~-7,682 bp</td>
<td>123 bp</td>
<td>TGCTTTTCCTCCAGCCTCT</td>
<td>ATTCACCTGGGGTCAACA</td>
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<td>146 bp</td>
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<td>TGATTTCAAGGCAACCCGA</td>
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<tr>
<td>Primer 5: -341~-281 bp</td>
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<td>TGGAAGTTGGCAAGGACT</td>
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<tr>
<td>Primer 6: -263~-108 bp</td>
<td>155 bp</td>
<td>AGGAGGTCAGTGGTGGTG</td>
<td>AGTGAGGCTGTTGGATTGT</td>
</tr>
</tbody>
</table>
Supplemental Figure 1

A

H3K4me3

relative enrichment (% input)

-8015~7793
-7805~7682
-454~308
-341~218
ChIP-qPCR Primers

B

H3K27me3

relative enrichment (% input)

-8015~7793
-7805~7682
-454~308
-341~218
ChIP-qPCR Primers

C

H3 acetylation

relative enrichment (% input)

-8015~7793
-7805~7682
-454~308
-341~218
ChIP-qPCR Primers

D

PXR

relative enrichment (% input)

-8015~7793
-7805~7682
-454~308
-341~218
ChIP-qPCR Primers

E

NCOA6

relative enrichment (% input)

-8015~7793
-7805~7682
-454~308
-341~218
ChIP-qPCR Primers

F

p300

relative enrichment (% input)

-8015~7793
-7805~7682
-454~308
-341~218
ChIP-qPCR Primers

Legend:

- DMSO control
- RIF 48 hrs
- RIF 96 hrs
- IgG

Significance:

* p < 0.05
** p < 0.01
Supplemental Figure 2

A

H3K4me3

relative enrichment (%/input)

-8015~7793 -7805~7682 -454~308 -341~218 ChIP-qPCR Primers

B

H3K27me3

relative enrichment (%/input)

-8015~7793 -7805~7682 -454~308 -341~218 ChIP-qPCR Primers

C

H3 acetylation

relative enrichment (%/input)

-8015~7793 -7805~7682 -454~308 -341~218 ChIP-qPCR Primers

D

H3K4me3

relative enrichment (%/input)

-8015~7793 -7805~7682 -454~308 -341~218 ChIP-qPCR Primers

E

H3K27me3

relative enrichment (%/input)

-8015~7793 -7805~7682 -454~308 -341~218 ChIP-qPCR Primers

F

H3 acetylation

relative enrichment (%/input)

-8015~7793 -7805~7682 -454~308 -341~218 ChIP-qPCR Primers

G

NCOA6

relative enrichment (%/input)

-8015~7793 -7805~7682 -454~308 -341~218 ChIP-qPCR Primers

H

p300

relative enrichment (%/input)

-8015~7793 -7805~7682 -454~308 -341~218 ChIP-qPCR Primers