The aldo-keto reductase *Akr1b7* gene is a common transcriptional target of xenobiotic receptors PXR and CAR

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Running Title: PXR and CAR regulate *Akr1b7*

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Number of text pages: 29

Number of figures: 5

Number of references: 40

Number of words in the abstract: 242

Number of words in the introduction: 623

Number of words in the discussion: 1198

Abbreviations: AKR, aldo-keto reductase; CAR, constitutive androstane receptor; DR, direct

repeat; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate

dehydrogenase; LXR, liver X receptor; MDA, malondialdehyde; PCN,

pregnenolone-16α-carbonitrile; PEI, polyethyleneimine; PUFA, polyunsaturated fatty acid;

PXR, pregnane X receptor; PXRE, PXR response element; VP, viral protein 16; RXR, retinoid

X receptor; TCPOBOP, 1,4-*bis*[2-(3,5-dichlorpyridyloxyl)]-benzene

ABSTRACT

AKR1B7 (aldo-keto reductase family 1, member 7), a member of the AKR superfamily, has been suggested to play an important role in the detoxification of lipid peroxidation byproducts. The nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are xenosensors postulated to alleviate xeno- and endobiotic chemical insults. In this study, we show that the mouse *Akr1b7* is a shared transcriptional target of PXR and CAR in the liver and intestine. Treatment of wild type mice with the PXR agonist pregnenolone-16α-carbonitrile (PCN) activated *Akr1b7* gene expression, whereas the effect was abrogated in PXR$^{-/-}$ mice. Similarly, the activation of *Akr1b7* gene expression by the CAR agonist 1,4-*bis*[2-(3,5-dichlorpyridyloxyl)]-benzene (TCPOBOP), seen in wild type mice, was abolished in CAR$^{-/-}$ mice. The promoter of *Akr1b7* gene was activated by PXR and CAR, and this activation was achieved through the binding of PXR-RXR or CAR-RXR heterodimers to a DR-4 type nuclear receptor-binding site found in the *Akr1b7* gene promoter. At the functional level, treatment with PCN in wild type, but not PXR$^{-/-}$, mice, led to a decreased intestinal accumulation of malondialdehyde (MDA), a biomarker of lipid peroxidation. The regulation of *Akr1b7* by PXR was independent of the liver X receptor (LXR), another nuclear receptor known to regulate this AKR isoform. Because a major function of Akr1b7 is to detoxify lipid peroxidation, the PXR-, CAR- and LXR-controlled regulatory network of *Akr1b7* may have contributed to alleviate toxicity associated with lipid peroxidation.

INTRODUCTION

The aldo-keto reductase (AKR) superfamily of genes encodes NAD(P)H linked oxidoreductases. AKRs play an important role in the detoxification of harmful aldehydes and ketones generated from exogenous and endogenous toxicants, and those produced from the breakdown of lipid peroxides. AKRs reduce aldehydes and ketones to their respective alcohols (Penning and Drury, 2007). Among AKR isoforms, the mouse *Akr1b7* is highly expressed in *vas deferens* and adrenal gland, where its sustained expression is dependent on androgen and adrenocorticotropic hormone, respectively (Lau *et al.* 1995). Interestingly, *Akr1b7* null mice were found to be viable and have no obvious defect in reproduction (Baumann *et al.* 2007). Besides the steroidogenic tissues, *Akr1b7* is also expressed in mouse kidney, eye, intestine, and, at a lower level, in liver (Lau *et al.* 1995).

One of the major functions of Akr1b7 is to detoxify lipid peroxidation. Lipid peroxidation refers to the oxidative deterioration of lipids containing carbon-carbon double bonds, especially those derived from polyunsaturated fatty acids (PUFAs). The peroxidation process proceeds by a free radical chain reaction, resulting in the production of many reactive aldehydes, among which the *trans*-4-hydroxy-2-nonenal (4-HNE) was recognized as having the greatest toxic and harmful potential (Schneider *et al.* 2008). All unsaturated aldehydes may undergo further changes by autoxidation, leading to the production of other volatile derivatives, such as malondialdehyde (MDA). Akr1b7 has a preference to the byproducts of lipid peroxidation as its substrates. These include 4-HNE (Schneider *et al.* 2008) and isocaproaldehyde, another highly toxic lipid byproduct generated during steroidogenesis (Lefrancois-Martinez *et al.* 1999).

The xenobiotic nuclear receptors, including pregnane X receptor (PXR, NR1I2)

(Kliewer *et al*., 1998; Blumburg *et al*., 1998) and constitutive androstane receptor (CAR,

NR1I3) (Honkakoski *et al*., 1998; Wei *et al*., 2000), were postulated to play an essential role in

the detoxification of xeno- and endo-biotic toxicants. The detoxifying effect of PXR and CAR

is achieved through the coordinate transcriptional regulation of Phase I and Phase II enzymes,

as well as drug transporters (for a review, see Timsit and Negishi, 2007). PXR and CAR

regulate gene expression by heterodimerization with the retinoic X receptor (RXR), and the

binding of the PXR-RXR or CAR-RXR heterodimers to specific response elements termed

PXR response elements (PXREs) or CAR response elements (also called phenobarbital

response element, or PBRE) that contain a hexanucleotide direct repeat separated by three or

four nucleotides (DR-3 or DR-4) (Honkakoski *et al*., 1998). In addition to its function in

xenobiotic detoxification, PXR has also been implicated in many other endobiotic functions,

ranging from bile acid detoxification and cholestatic prevention (Xie *et al.* 2001; Staudinger *et

al.* 2001), to bilirubin detoxification and clearance, adrenal steroid homeostasis and

drug-hormone interactions (Zhai *et al.* 2006), lipid metabolism (Zhou J. *et al*, 2006; Nakamura

et al. 2007; and for a review, see Handschin and Meyer, 2005), inflammation and

inflammatory bowel disease (Langmann *et al.* 2004; Zhou C *et al.* 2006; Dring *et al.* 2006;

Shah *et al.* 2007), bone homeostasis (Pascussi *et al.* 2005), and retinoid acid metabolism

(Wang *et al.* 2008) (for a review, see Zhang *et al.* 2008). Compared to its sister PXR, CAR

exhibits many overlapping, yet distinct, functions (Timsit and Negishi, 2007). The liver X

receptor (LXR), another nuclear hormone receptor, has been shown to regulate *Akr1b7* (Volle

et al. 2004). However, there has been no report on the role of PXR or CAR in the regulation of

AKRs and detoxification of lipid peroxidation.

In this report, we show that *Akr1b7* is a likely transcriptional target of PXR and CAR,

suggesting a novel role for xenobiotic receptors in the detoxification of lipid peroxidation. The

regulatory network of *Akr1b7* controlled by PXR, CAR and LXR may provide a complex and

fail-safe system in preventing toxicity associated with lipid peroxidation.

MATERIALS AND METHODS

Animals and drug treatment. The PXR null (PXR$^{-/-}$) (Xie *et al.* 2000), CAR null (PXR$^{-/-}$) (Wei *et al.*, 2000), LXR α and β double knockout (LXR DKO) (Peet *et al.*, 1998) and FABP-VP-PXR transgenic (Gong *et al*, 2006) mice in C57BL/6J and SvJ129 mixed background have been described previously. Mice were housed in a pathogen-free animal facility under a standard 12 hrs light/dark cycle with free access to water and food. Age- and sex-matched 8-10 weeks old mice were used for all the experiments. To activate PXR, mice received two (for gene expression analysis) or four (for lipid peroxidation analysis) daily i.p. injections of PCN (100 mg/kg) and were sacrificed 4 hrs after the last dose. To activate LXR, mice received daily gavages of GW3965 (20 mg/kg) for 5 days and were sacrificed 24 hrs the last dose. To activate CAR, mice received a single i.p. injection of TCPOBOP (1 mg/kg) and were sacrificed 7 days after the injection. PCN and TCPOBOP were purchased from Sigma. GW3965 was synthesized in-house following the published scheme (Zhou *et al*., The use of mice in this study has complied with all the relevant federal guidelines and institutional policies.

Real-time RT-PCR. Total RNA was isolated by using TRIZOL® reagent from Invitrogen (Carlsbad, CA). Reverse transcription was performed by using iScript cDNA Synthesis Kit from Bio-Rad (Stanford, CA, USA). Real-time PCR was performed with the 7300 Real-Time PCR system (Applied Biosystems, CA) by using the SYBR-green reagents. All the data were normalized against the mouse *cyclophilin* gene. The primer sequences of *Akr1b7* are, forward: 5'-CCCTCACGCATACAGGAGAA-3' and reverse: 5'-GCCATGTCCTCCTCACTCAA-3'

(Volle *et al.* 2004). Other real-time PCR primers are: *Akr1a4*, forward:

5'-GCTTAGATGGCAGGTTCAGC-3' and reverse: 5'-AGCATCTCTGGGAACCCTCT-3';

Akr1b8, forward: 5'-CCTCACCCAGGAAAAACTGA-3' and reverse:

5'-CACGTTCCTCTGGATGTGAA-3'; *Akr7a5*, forward:

5'-ATCAGGAGGGCAAGTTTGTG-3' and reverse 5'-CCAAAGGGTTGTAGGCGTAG-3'.

Northern blot analysis. The *Ark1b7* cDNA probe was amplified by PCR from mouse liver

cDNA by using the primers: forward 5'-CAATGAGAATGAGGTGGGAG-3', and reverse

5'-CCTCACTCAACTGGAAGTCGA-3'. The *Cyp3a11* cDNA probe was previously

descried (Xie *et al.* 2000). Northern blot analysis was performed as described previously (Xie

et al. 2000). *Gapdh* cDNA probing was used for the loading control.

Plasmid constructs and transfection assays. The 5'-regulatory sequence (-1972 bp to -4 bp)

of the mouse *Akr1b7* gene was cloned by PCR using the following pair of oligonucleotides:

forward, 5'-GGTACCCATGTTCTAGTCAGCCATGGC-3' (engineered with *KpnI* site); and

reverse primer, 5'-CTCGAGGTGAGCAGATGAAATGCCTG-3' (engineered with *XhoI* site).

The design of oligonucleotides was based on mouse genomic sequences deposited in

GenBank[TM] (accession number NC_000072). The mouse liver genomic DNA was used as

the PCR template. The PCR-amplified fragments were cloned into the pGL-3 basic vector

form Promega (Madison, WI). Site-directed mutagenesis was performed by the PCR

overextension method and the mutations were confirmed by DNA sequencing.

Transient transfection was performed in HepG2 cells seeded in 48-well plates with

polyethyleneimine (PEI) polymer as the transfection reagent (Zhou J *et al.* 2006). For each

well, the plasmid-PEI complexes were formed by incubating 0.1 µg of expression vector for

each nuclear receptor, 0.3 µg of reporter plasmid, 0.1 µg of β-galactosidase plasmid, and 0.5

µg of PEI at room temperature for 20 min in a total volume of 100 µl of serum-free

Dulbecco's Modified Eagle's Medium (DMEM). The DNA-PEI complexes were then diluted

with 100 µl of DMEM and applied at 200 µl per well. After 12 hrs of incubation, the

transfection medium was replaced with DMEM containing 10% fetal bovine serum with the

appropriate solvent or ligands. Cells were lysed and assayed for luciferase and

β-galactosidase after 24 hrs of drug treatment.

Identification of the putative PXR/CAR response elements and electrophoretic mobility

shift assay (EMSA). The promoter sequence analysis was performed by using the web-based

software NUBIScan V2.0 (http://www.nubiscan.unibas.ch/). The putative response elements

were then analyzed for receptor binding by EMSA. For the EMSA assay, receptor proteins

were prepared by using the *in vitro* TNT® quick coupled transcription/translation system

from Promega. The nucleotide probes were labeled with ^{32}P-dCTP by Klenow fill-in method.

The binding reactions were performed at room temperature for 20 min (Wada *et al.* 2008).

Protein-DNA complexes were resolved by vertical electrophoresis through 8%

polyacrylamide gel in 0.5× TBE at 4°C for 1.5 hrs. The gel was then dried and proceeds for

autoradiograph. The probe sequences are labeled in the figure. Unlabeled competitor DNAs

(50-100×) were used for the parallel competition experiments.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed essentially as previously described (Wada et al., 2008). In brief, primary mouse hepatocytes were prepared from wild type mice by the colleagenase perfusion method. Cells were treated with solvent (DMSO) or PCN (10 μM) for 24 hrs before formaldehyde crosslinking. Cell lysates were incubated overnight with 1 μg of anti-PXR antibody (Cat # sc-7737) from Santa Cruz (Santa Cruz, CA) at 4 ^{o}C. Parallel samples were incubated with normal IgG as a negative control. The following PCR primers were used: *Akr1b7*–Forward, 5'-ATATCCACTCCCAGGGCAAT-3'; *Akr1b7*–Reverse, 5'-GGGCATGAGAACAGGAACTT-3'. The PCR primers for the positive control *Cd36* gene (Zhou et al., 2006) and negative control *Cyp7b1* gene (Wada et al., 2008) were previously described.

Intestinal malondialdehyde (MDA) measurement. Freshly dissected mouse small intestine samples were homogenized on ice in KCl (150 mM) solution (1 g wet tissue in 9 ml KCl) using a Polytron homogenizer. For each assay, 100 μl of the supernatant was incubated with 900 μl reaction buffer containing 0.67% thiobarbituric acid (TBA). This mixture was incubated at 98°C for 1 hr, and then centrifuged at 12,000×g for 10 min at 4°C. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The absorbance was converted to nmol MDA from a standard curve generated with 1,1,3,3-tetramethoxypropane.

Western blot analysis. Duodenum was collected from mice and homogenized in lysis buffer

(50 mM Tris (pH7.4), 1% NP-40, 0.25% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM

EDTA, 1 mM PMSF, 1 mM NaF, and 0.2% protease inhibitor cocktail from Sigma (St Louis,

MO). The lysates were centrifuged at 13000×g for 20 min at 4°C and the supernatants were

collected. The protein concentrations in the supernatants were measured. Equal amounts of

lysate proteins were resolved by 10% SDS-PAGE gel, and then transferred to

polyvinylidinedifluoride membrane. Membranes were blocked with 5% non-fat dry milk in

TBS-Tween 20 at room temperature for 1 hr. Akr1b7 protein was detected by a primary

polyclonal goat antibody against Akr1b7 (Cat # sc-27763, 1:500) from Santa Cruz and a

secondary peroxidase-conjugated donkey anti-goat IgG antibody (Cat # sc-2020, 1:2000)

from Santa Cruz. The control β-actin protein was detected by a primary monoclonal mouse

antibody against β-actin (1:5000; Sigma) and a secondary peroxidase-conjugated horse

anti-mouse IgG antibody (1:4000; Cell Signaling Technology, Danvers, MA). Protein signals

were detected with the enhanced chemiluminescence (ECL) reagents from Amersham

(Piscataway, NJ).

Statistical Analysis. Results are expressed as means ± SD. Statistical analysis was performed

using the unpaired student's *t* test for comparison between two groups.

RESULTS

Activation of PXR induced the expression of *Akr1b7 in vivo*

As shown in Figure 1, treatment of wild type mice with the PXR agonist PCN for 28 hrs induced the mRNA expression of *Akr1b7* in the liver and small intestine, as determined by Northern blot analysis (Fig. 1A). *Cyp3a11*, a known PXR target gene, was included as a positive control for PXR activation. A similar *Akr1b7* gene activation was observed in mice treated with PCN for 52 hrs (data not shown). The induction of intestinal Akr1b7 protein expression by PCN was also confirmed by Western blot analysis. (Fig. 1B). The effect of PCN on *Akr* gene expression appeared to be isoform-specific, because the expression of *Akrs 1a4*, *1b8* and *7a5* in the same animals was not significantly altered (Fig. 1C). The PCN effect on *Akr1b7* gene expression was completely abolished in PXR$^{-/-}$ mice (Xie *et al*., 2000) (Fig. 1D), demonstrating that PXR is the *bona fide* mediator for the PCN effect. We have previously reported the creation of FABP-VP-PXR transgenic mice in which a constitutively activated human PXR (VP-PXR) was expressed in the liver and intestine under the control of the rat fatty acid binding protein (FABP) gene promoter (Gong *et al,* 2006). Figure 1E showed that the *Akr1b7* gene activation was also observed in FABP-VP-PXR transgenic mice, although the magnitude of gene regulation appeared to be not as dramatic as that in the PCN-treated wild type mice. In the same FABP-VP-PXR transgenic mice, the expression of *Cyp3a11* was induced in both the liver and intestine (Gong *et al.* 2006). Interestingly, we found that the basal and PXR inducible expression of *Akr1b7* was higher in the intestine than that in the liver in both the pharmacological and genetic models of PXR activation. These results strongly suggest that *Akr1b7* is a transcriptional target of PXR.

Activation of CAR induced the expression of *Akr1b7 in vivo*

We found that treatment of wild type mice with the CAR agonist TCPOBOP also

induced the mRNA expression of *Akr1b7* in the liver (Fig. 2A) and small intestine (Fig. 2B).

Cyp2b10, a prototypical CAR target gene, was included as a positive control for CAR

activation. The TCPOBOP effect on *Akr1b7* gene expression in the liver (Fig. 2C) and small

intestine (Fig. 2D) was abolished in CAR$^{-/-}$ mice (Wei *et al.*, 2000), suggesting that *Akr1b7* is

also under the positive control of CAR. There was an additive induction of *Akr1b7* mRNA

expression in mice treated with both PCN and TCPOBOP (Fig. 2E).

**The *Akr1b7* gene promoter was activated by PXR and CAR and identification of the
response element in the promoter region of *Akr1b7* that binds to both PXR and CAR**

To understand the molecular mechanism by which PXR and CAR regulate *Akr1b7*

gene expression, we cloned the 2-kb 5' flanking region of the mouse *Akr1b7* gene by PCR.

Inspection of the promoter sequences and assisted by using the web-based software

NUBIScan V2.0 (http://www.nubiscan.unibas.ch/), we have identified three candidate DR-4

(DR4-1, 2 and 3) and one DR-3 site (Fig. 3A) within 449-bp upstream of the transcription

start site. After an initial screening by EMSA, we found one of the DR-4 elements, DR4-3,

had a strong affinity to bind to the PXR-RXR heterodimers (Fig. 3B). The binding can be

efficiently competed by unlabeled Akr1b7/DR-4 or the MDR-1/DR-4

(AGGTCAagttAGTTCA) derived from the MDR-1 gene (Geick *et al.* 2001), but not by two

Akr1b7/DR-4 mutant variants with the DR-4 site disrupted (Fig. 3A and 3B). A similar

pattern of binding and competition was observed when the activated VP-PXR was used in

EMSA (data not shown). The *in vivo* recruitment of PXR to DR4-3 was confirmed by

chromatin immunoprecipitation (ChIP) assay. As shown in Fig. 3C, treatment of primary

hepatocytes with PCN resulted in the recruitment of PXR to DR4-3. In the ChIP assay, *Cd36*

(a PXR target gene) (Zhou et al., 2006) and *Cyp7b1* (a RORα target gene) (Wada et al., 2008)

were included as positive and negative control, respectively (Fig. 3C). EMSA showed that

DR4-1 and DR4-2, but not DR-3, could also bind to the PXR-RXR heterodimers (data not

shown). Akr1b7/DR-4 can also specifically bind to CAR-RXR heterodimers as shown by

EMSA (Fig. 3D).

To examine the functional relevance of DR-4s in mediating the transactivation by

PXR and CAR, the 2-kb 5' regulatory DNA fragment was inserted into the pGL3 basic vector

to create the pGL3-Akr1b7 luciferase reporter gene. Three variant reporter genes with three

DR-4s individually mutated were also generated. In transient transfection and luciferase

reporter gene assay, pGL3-Akr1b7 was activated by PCN in HepG$_2$ cells co-transfected with

the expression vector for the mouse PXR (pCMX-mPXR) (Fig. 3E). Interestingly, the

mutation of any of the DR-4s resulted in the loss of PXR effect (Fig. 3E). When the mouse

CAR (pCMX-mCAR) was co-transfected, we found that CAR activated the wild type report

gene in the absence of an exogenously added ligand, and this activation was enhanced when

TCPOBOP was added to the medium (Fig. 3E). Similarly, the mutation of any of the DR4

sites abolished the effect of CAR and TCPOBOP (Fig. 3E).

Independent and cooperative regulation of *Akr1b7* by PXR and LXR

Since liver X reporter α (LXRα) has previously been shown to regulate *Akr1b7* via

several LXREs including the DR-4 that binds to PXR and CAR (Volle *et al.* 2004), we went

on to determine whether the effects of PXR and LXR on *Akr1b7* gene expression are

mutually dependent. As shown in Fig. 4A, PCN was effective to induce *Akr1b7* mRNA

expression in the liver of LXR α and β double knockout (LXR DKO) mice (Peet *et al.*, 1998).

Conversely, GW3965, a LXR agonist that does not activate PXR (Zhou et al., 2008),

remained effective to induce *Akr1b7* gene expression in the liver of PXR$^{-/-}$ mice (Fig. 4B).

The expression of Scd-1, a known LXR target gene, was also induced in GW3965-treated

PXR$^{-/-}$ mice (data not shown). These results suggest that PXR and LXR are mutually

dispensable in their regulation of *Akr1b7* gene expression. To determine whether PXR and

LXR had an additive effect on *Akr1b7* gene expression, HepG$_2$ cells were transfected with

pGL3-Akr1b7 2-kb reporter gene together with expression vectors for PXR and/or LXR.

Transfected cells were then treated with PXR and/or LXR agonists for 24 hrs before

luciferase assay. As shown in Fig. 4C, PXR and LXR apparently had an additive effect in

activating the reporter gene.

A potential role of PXR in protection against lipid peroxidation

Akr1b7 has been suggested to play an important role in the detoxification of lipid

peroxidation products (Schneider *et al.* 2008; Volle *et al.* 2004). To determine whether PXR

activation confers protection from lipid peroxidation, we analyzed lipid peroxidation in the

small intestine of mice by measuring the level of malondialdehyde (MDA), a byproduct of

polyunsaturated fatty acid peroxidation (Frankel, 1987) and biomarker of lipid peroxidation.

A significant decrease in MDA level was observed in wild type mice treated with PCN for 52

hrs, as compared to the vehicle treated counterparts (Fig. 5A). The PCN effect on MDA

production was abolished in PXR^{-/-} mice (Fig. 5B). These results suggest that PXR plays a

role in alleviating lipid peroxide accumulation in the small intestine. When the CAR effect

was evaluated, we were surprised to find that treatment of TCPOBOP had little effect on the

basal level of MDA in either the wild type (Fig. 5C) or CAR^{-/-} (Fig. 5D) mice.

DISCUSSION

In this study, we have identified *Akr1b7* as a novel target gene for the xenobiotic receptors PXR and CAR. The combined uses of pharmacologic and genetic models of PXR and CAR activation have demonstrated that activation of PXR and CAR is both necessary and sufficient for the regulation of *Akr1b7* in the mouse liver and intestine. The identity of *Akr1b7* as a PXR and CAR target gene was further supported by the characterization of a DR-4 response element that binds to both receptors and is required for the transactivation of *Akr1b7* gene promoter. The activation of *Akr1b7* gene expression by PXR and CAR has led to our appreciation that the xenobiotic receptors may have a previously unrecognized function in the detoxification of lipid peroxides.

Both RXR and LXR agonists have been shown to activate *Akr1b7* gene expression. It was reported that the effect of RXR agonist LG268 on A*kr1b7* gene expression was intact in LXR DKO mice (Volle *et al.* 2004), suggesting that RXR heterodimerization partners other than LXRs could have mediated the transactivation. Our results suggested that PXR and CAR might have mediated the activation of *Akr1b7* gene expression by LG268 in LXR DKO mice. T0901317 was used by Volle and colleagues as the LXR agonist to induce *Akr1b7* gene expression (Volle *et al.* 2004). Interestingly, the effect of T0901317 on *Akr1b7* gene expression was completely abolished in LXR α and β double knockout mice (Volle *et al.* 2004), despite the reports that T0901317 can also activate PXR (Shenoy *et al.*, 2004).

Having demonstrated that both PXR and LXR activate *Akr1b7* gene expression, an outstanding question is whether the effect of these two receptors on *Akr1b7* gene expression is mutually dependent. Using mice deficient of PXR or LXRs, we showed that the PXR and LXR

agonist effect on *Akr1b7* gene expression was intact in LXR DKO and PXR$^{-/-}$ mice,

respectively. The independent effect of PXR and LXR was further supported by our

observation that PXR and LXR had an additive effect in activating the *Akr1b7* gene promoter.

The co-regulation of *Akr1b7* is reminiscent of the shared regulation of Sult2a1/2a9 by PXR

(Sonoda *et al.*, 2002), CAR (Saini *et al.*, 2004), FXR (Song *et al.*, 2001) and LXR (Uppal *et al.*,

2007), in which an inverted repeat without a spacing nucleotide (IR-0) response element is

used by all four receptors. We propose that the regulatory network of *Akr1b7*, controlled by

PXR, CAR and LXR, offers a complex and fail-safe system in preventing toxicity associated

with lipid peroxidation. It remains to be determined whether the shared regulation of *Akr1b7*

by several nuclear receptors also contributes to the tissue distribution pattern of this AKR

isoform.

The identification of *Akr1b7* as a PXR and CAR target gene has expanded the

function of xenobiotic receptors in xeno- and endobiotic detoxification. Since AKRs

functionalize carbonyl groups by forming alcohols for the conjugation reactions catalyzed by

the Phase II UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) enzymes, they

can be classified as Phase I enzymes (Penning and Drury, 2007). Human AKRs have been

implicated in the metabolism (carbonyl reduction) of synthetic hormones, cancer

chemotherapeutics, and central nervous system (CNS)-acting drugs. AKRs also play an

important xenobiotic role, implicating in the detoxification of at least three classes of

chemical carcinogen: polycyclic aromatic hydrocarbons, aflatoxin and nicotine derived

nitosamino-ketones (Penning and Drury, 2007). It remains to be determined whether the

human AKRs are also regulated by xenobiotic receptors. There was no report of the human

homolog of *Akr1b7*. *AKR1B10* appeared to be the closest human relative based on the AKR

family tree. Like Akr1b7, AKR1B10 is also highly expressed in the intestine and liver, and

functions as an aldose reductase (Cao et al., 1998). Using the human colon cancer LS180

cells overexpressing the wild type or activated PXR (Gong et al., 2006), we showed that

activation of PXR did not alter the expression of *AKR1B10* (data not shown). It remains to be

determined whether other human AKR isoforms can be regulated by PXR or CAR.

Although our results suggest that *Akr1b7* is under the transcriptional control of both

PXR and CAR, it appeared that there were differences between these two receptors in their

regulation of *Akr1b7*. For example, the magnitude of *Akr1b7* induction was higher in the

intestine than that in the liver in PXR-activated mice (Fig. 1); whereas the induction was

more dramatic in the liver than that in the intestine in CAR-activated mice (Fig. 2). The

mechanism for this tissue effect of *Akr1b7* gene regulation is currently unclear. Moreover,

unlike the PXR agonist PCN that inhibited MDA formation in the intestine in a

PXR-dependent manner (Fig. 5A and 5B), treatment with the CAR agonist TCPOBOP failed

to inhibit MDA formation (Fig. 5C and 5D). The receptor-specific effect on MDA formation

may have resulted from the differential effect of these two receptors on the expression of

other genes, whose products might also be involved in the production and clearance of lipid

peroxidation products.

The identification of *Akr1b7* as a PXR target gene has also expanded the function of

this receptor in lipid homeostasis. Originally identified as a "xenobiotic receptor," PXR was

later found to impact lipid homeostasis (Zhou J *et al.*, 2006; Nakamura *et al.* 2007; and for a

review, see Handschin and Meyer, 2005). The activation of PXR in the mouse liver resulted

in an increased hepatic deposit of triglycerides. This PXR-mediated lipid accumulation was

independent of the activation of the lipogenic transcriptional factor sterol regulatory

element-binding protein 1c (SREBP-1c). Instead, the PXR-responsive lipid accumulation

was associated with an increased expression of the free fatty acid transporter CD36 and

several accessory lipogenic enzymes, such as stearoyl CoA desaturase-1 (SCD-1) and long

chain free fatty acid elongase (FAE). In the same study, CD36 was established as a direct

transcriptional target of PXR. Increased fatty acid uptake, such as that facilitated by CD36,

may prone cells to lipid peroxidation. Indeed, an accumulation of polyunsaturated fatty acids

(PUFAs) have been proposed to be involved in the formation of atheromas in the vasculature,

because PUFAs serve as substrates for lipid peroxidation (Yin and Porter, 2005). It is

tempting for us to speculate that the activation of *Akr1b7* by PXR may represent an evolved

function for cells to protect from lipid peroxidation associated with increased fatty acid

uptake. Although we have shown that activation of PXR was necessary and sufficient to

decrease intestinal accumulation of the lipid peroxidation biomarker MDA, we cannot

excluded the possibility that the MDA-lowering effect of PXR may have been mediated or

contributed by PXR target genes other than Akr1b7. A future use of *Ark1b7* null mice

(Baumann et al., 2007) would further conclude that the PXR effect on the alleviation of lipid

peroxidation is indeed mediated by this enzyme. Also interesting is that Akr1b7 has been

suggested to affect lipid metabolism by inhibiting adiopogenesis in some adipose tissues, the

mechanism of which remains to be determined (Tirard et al., 2007).

    In summary, the present work has established a novel anti-lipid peroxidation role for

the xenobiotic receptors PXR and CAR. The regulation of *Akr1b7* has not only expanded the

detoxification function of PXR, but also broadened the implications of xenobiotic receptors

in lipid homeostasis.

ACKNOWLEDGEMENTS

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Footnotes:

FIGURE LEGENDS

Figure 1. Activation of PXR induced the expression of *Akr1b7 in vivo*.

(A) The expression of *Akr1b7* mRNA in mouse liver and small intestine was measured by

Northern blot analysis. Male mice were treated with two daily i.p. doses of PCN (100 mg/kg)

and sacrificed 4 hrs after the second dose (the total treatment time is 28 hrs). C*yp3a11* was

included as a positive control for PXR activation. Membranes were stripped and re-probed

for glyceraldehyde-3-phosphate dehydrogenase (Gapdh) for a loading control. Lanes

represent individual mice. Note four and three PCN-treated mice were used for the liver and

intestinal analysis, respectively. (B) Expression of Akr1b7 protein as determined by Western

blot analysis. Lanes represent individual mice. (C) The expression of *Akr1b7*, *Akr1a4*,

Akr1b8 and *Akr7a5* mRNA in the liver was measured by real-time PCR. The same samples in

(A) were used. (D) The hepatic and intestinal mRNA expression of *Akr1b7* in male PXR$^{-/-}$

mice treated without or with PCN was measured by real-time PCR. N=6 for each group. (E)

The mRNA expression of *Akr1b7* in the liver and small intestine of wild type (WT) mice and

FABP- VP-PXR transgenic (TG) mice was measured by Northern blot analysis. Each lane

represents RNA pooled from three mice. NS, statistically not significant (*P*>0.05).

Figure 2. Activation of CAR induced the expression of *Akr1b7 in vivo*.

(A and B) Real-time PCR analysis on the mRNA expression of *Akr1b7* in the liver (A) and

small intestine (B) of mice treated with vehicle or TCPOBOP (single i.p. dose of 1 mg/kg,

mice were sacrificed 7 days after injection). C*yp2b10* was included as a positive control for

CAR activation. N=5 for each group. (C and D) The hepatic (C) and intestinal (D) expression

of *Akr1b7* and *Cyp2b10* was analyzed in vehicle- and TCPOBOP-treated CAR^{-/-} mice. (E)

Hepatic expression of *Akr1b7* mRNA in mice treated with both PCN and TCPOBOP. N=5 for

each group. **, $P<0.01$, compared to the vehicle groups; NS, statistically not significant

($P>0.05$).

Figure 3. The *Akr1b7* gene promoter was activated by PXR and CAR and identification

of the response element in the promoter region of *Akr1b7* that binds to both PXR and

CAR.

(A) Schematic diagram of the mouse *Akr1b7* gene promoter. Numbers indicate positions

from the transcriptional starting site (+1). Three putative DR-4 elements and one DR-3

element are labeled. The sequences of DR4-1 and DR4-2 mutants and two DR4-3 mutants

are also labeled with the mutated nucleotides underlined. (B) Binding of the PXR-RXR

heterodimers to the radio-labeled Akr1b7/DR4-3. The arrows indicate specific shift bands. In

the competition lanes, the unlabeled competitor oligoes were added at 50-100 folds in excess.

Akr and MDR indicate Akr1b7/DR4-3 and MDR1/DR-4, respectively. (C) Recruitment of

PXR to Akr1b7/DR4-3 as shown by ChIP assays. *Cd36* and *Cyp7b1* were included as the

positive and negative control of PXR target genes, respectively. (D) CAR-RXR heterodimers

bind to Akr1b7/DR-4. The dark and light arrows indicate specific and non-specific shift

bands, respectively. (E) Wild type (WT) *Akr1b7* 2-kb promoter reporter gene or its DR4

mutant variants were co-transfected with the mouse PXR expression vector (pCMX-mPXR)

or the mouse CAR expression vector (pCMX-mCAR) into HepG2 cells. 12 hrs after

transfection, cells were treated with vehicle (DMSO), PCN (10 µM), or TCPOBOP (250 nM)

for 24 hrs before luciferase assay. The luciferase activity was normalized against the

co-transfected β-galactosidase activity. Results are shown as fold induction over vehicle

controls and represent the average and standard deviation from triplicate assays.

Figure 4. Independent and cooperative regulation of *Akr1b7* by PXR and LXR.

(A) *Akr1b7* mRNA expression was induced by the PXR agonist PCN in LXR α and β double

knockout (LXR DKO) mice. LXR DKO mice were treated with vehicle or PCN and the liver

expression of *Akr1b7* was analyzed by real-time PCR. N=5 for each group. (B) *Akr1b7*

mRNA expression was induced by the LXR agonist GW3965 in $PXR^{-/-}$ mice. $PXR^{-/-}$ mice

were treated with five daily gavages of vehicle or GW3965 (20 mg/kg). The liver expression

of *Akr1b7* was analyzed by Northern blot analysis. Lanes represent individual mice. (C) The

pGL3-Akr1b7 2-kb reporter gene was co-transfected with PXR and/or LXR. Transfected

cells were treated with the indicated drugs for 24 hrs before luciferase assay. The drug

concentration is 10 μM for both PCN and GW3965.

Figure 5. A potential role of PXR in protection against lipid peroxidation.

(A and B) The level of malondialdehyde (MDA) in the small intestine homogenate was

measured in WT (A, N=4 for each group) or $PXR^{-/-}$ (B, N=3 for each group) mice treated

with DMSO or PCN. Mice received four daily i.p. injections of the drugs and sacrificed 4 hrs

after the last dose, so the total treatment time is 76 hrs. (C and D) The intestinal MDA level

was measured in WT (C, N=5 for each group) or $CAR^{-/-}$ (D, N=5 for each group) mice

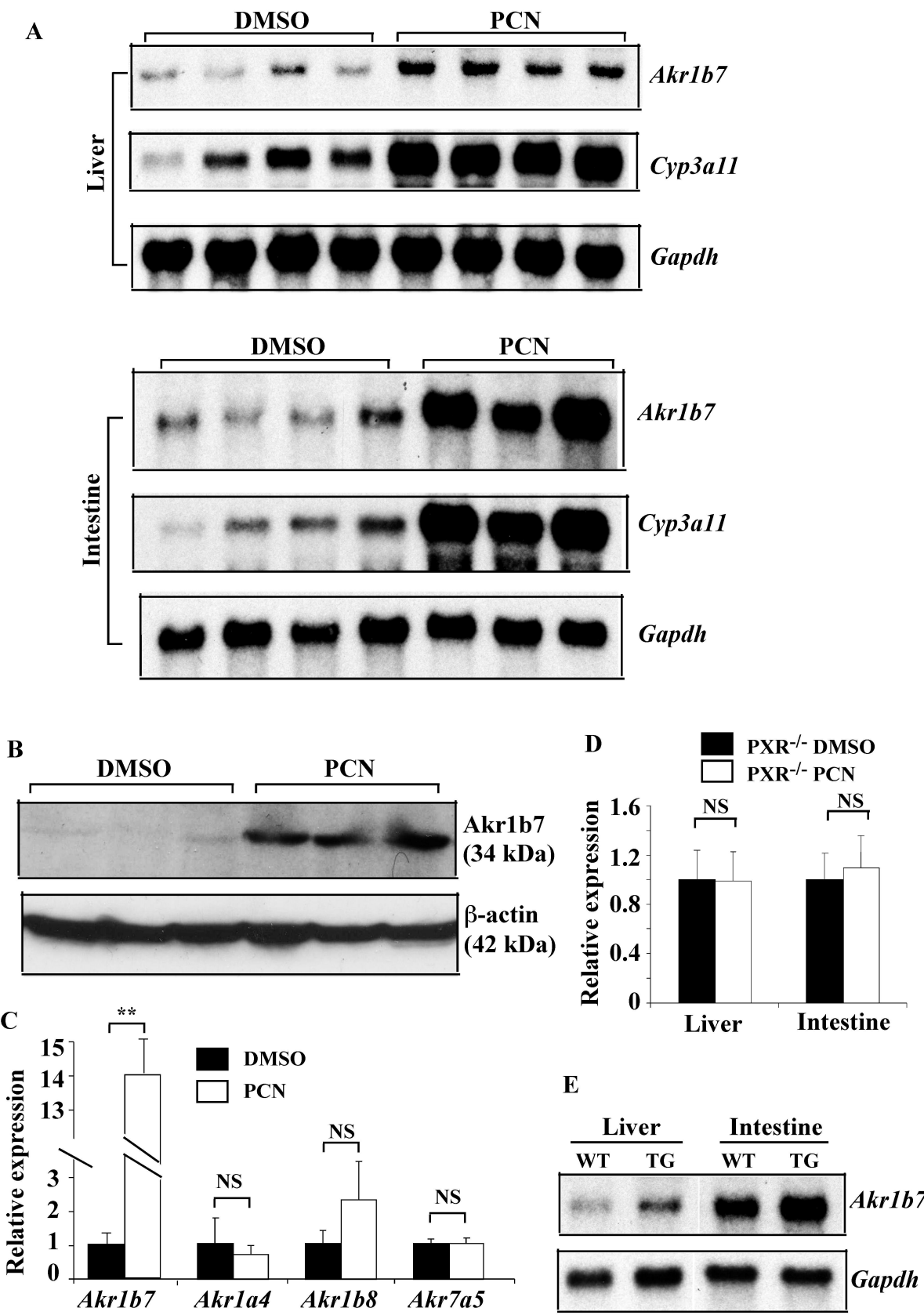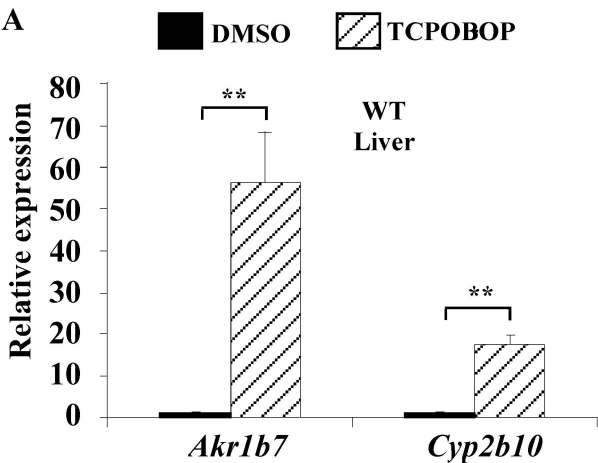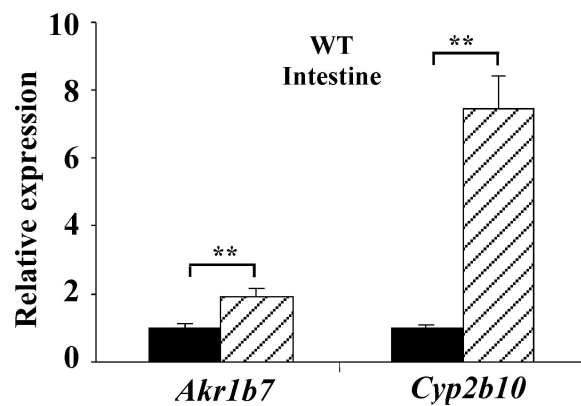treated with DMSO or TCPOBOP. *, $P<0.05$; NS, statistically not significant ($P>0.05$).

Fig. 1

Fig. 2

Fig. 3

A

DR4-3 mut: ATATAActtaGATTAA

DR4-3 mut1: ATATAActtaGGTTCA

-263 DR4-3 -248
ATGTCActtaGGTTCA

-194 DR4-2 -179
TGCACTgtgcTGACTT

-2-kb   +1

AGGACAgttATTTCA
-435 DR3 -421

TCATCTtggaTGACCT
-286 DR4-1 -271

DR4-1 mut: TCGTGTtggaTGCAAT

DR4-2 mut: TACAATgtgcTATGTT

B

Competitor: - - - Self MDR Mut Mut1
PXR: + - + + + + + + +
RXR: - + + + + + + + +

³²P-Akr1b7/DR4-(3)

C

Input   IgG   Akr1b7

Akr1b7

Cd36

Cyp7b1

D

Competitor: - - - Akr MDR Mut Mut1
CAR: + - + + + + +
RXR: - + + + + + +

(non-specific)

³²P-Akr1b7/DR4-(3)

E

Fold Activation

- ■ Akr1b7 2kb (WT)
- ▨ Akr1b7 2kb (DR4-1m)
- □ Akr1b7 2kb (DR4-2m)
- ▨ Akr1b7 2kb (DR4-3m)

DMSO   PCN   DMSO   DMSO   TCPOBOP
pCMX-mPXR   Vector   pCMX-mCAR

Fig. 4

Fig. 5