HORIZON DISCOVERY

P450 INDUCTION IN CRYOPRESERVED HEPATOCYTES FROM PXR AND CAR NUCLEAR RECEPTOR KNOCK-OUT RATS

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Abstract

The nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are closely related transcription factors that regulate the expression of phase I (cytochrome P450s), phase II metabolizing enzymes and transporter genes in response to xenobiotics, including prescription drugs. Here, we report the isolation and preliminary characterization of cryopreserved hepatocytes from male PXR and CAR knockout (SD) rats and PXR/CAR double knockout (SD) rats (Horizon Discovery - SAGE Labs). We were successful in the isolation and cryopreservation of the hepatocytes from the three knockout models, yielding hepatocytes with high (>80%) viability and plateability. The cryopreserved hepatocytes from wildtype and knockout rats were cultured for the evaluation of gene expression in the presence and absence of PXR and CAR ligands. The cryopreserved hepatocytes were recovered using UCRM (IVAL Inc.) and plated in 24-well collagen-coated plates. The hepatocytes formed near confluent monolayer cultures with epithelial morphology typical of primary cultured rat hepatocytes. After culturing overnight, medium was changed to protein free induction medium for rat hepatocytes (RHIM, IVAL Inc.). The hepatocytes were treated the next day with the PXR-ligand pregnenlone-16α-carbonitrile (PCN) and the CAR-ligand 3,3′,5,5′-tetrachloro-1,4-bis(pyridyloxy) benzene (TCPOBOP). Our results show that PXR was required for PCN-activation of Cyp2b2, Cyp3a23/3a1, Cyp3a18, Cyp2c11 and Slco1a2 (Oatp1a2) gene expression, and that CAR was required for TCPOBOP-activation of Cyp2b2 and Cyp3a23/3a1 gene expression, in good agreement with data obtained from *in vivo* rat knockout models[1], human primary hepatocytes, nuclear receptor knock-out cell lines and mouse knock-out models. Hepatocytes from the PXR and CAR knockout rats therefore represent a useful *ex vivo* complement to the *in vivo* models and tools for drug development, especially for functional studies on metabolic pathways involving nuclear receptors.

Materials and Methods

Hepatocyte Reagents and Materials:
IVAL Plateable Cryopreserved Rat Hepatocytes:
Male SD, Cat#: 82018, Lot# RSM111 (age, 7-10 weeks)
Male PXR KO, Cat#: 82169, Lot#: RS133 (age, 7 weeks)
Male CAR KO, Cat#: 82114, Lot#: RS132 (age, 10 weeks)
Male PXR/CAR KO, Cat#: 82165, Lot#: RS134 (age, 8 weeks);
UCRM™ - Universal Cryopreservation Recovery Medium, 50 mL, iVAL Cat#: 81015
UPCM™ - Universal Primary Cell Plating Medium, 50 mL, iVAL Cat#: 81016
RHIM™ - Rodent Hepatocyte Induction Medium, 500 mL (with supplement),
iVAL Cat#: 81038
CellAffix™ Collagen I Coated Plate, 24-well, 5/pack, APS Cat#: 71006

Hepatocyte Thawing Counting and Plating Procedure:
Follow iVAL's protocol for Thawing, Counting and Plating Cryopreserved plateable Hepatocytes. In short, thaw and recover rat hepatocytes with UCRM and plate for 4-6 hours in UPCM. Replace UPCM with RHIM and proceed to compound treatment.

We plated 350,000 hepatocytes of each line in 24-well CellAffix Collagen I Coated Plates 9-replicates. Three biological replicates for each treatment.

Chemicals.
Pregnenolone-16α-Carbonitrile (PCN) and 3,3′,5,5′-tetrachloro-1,4-bis (pyridyloxy) benzene (TCPOBOP), were obtained from Sigma-Aldrich (St. Louis, MO USA),

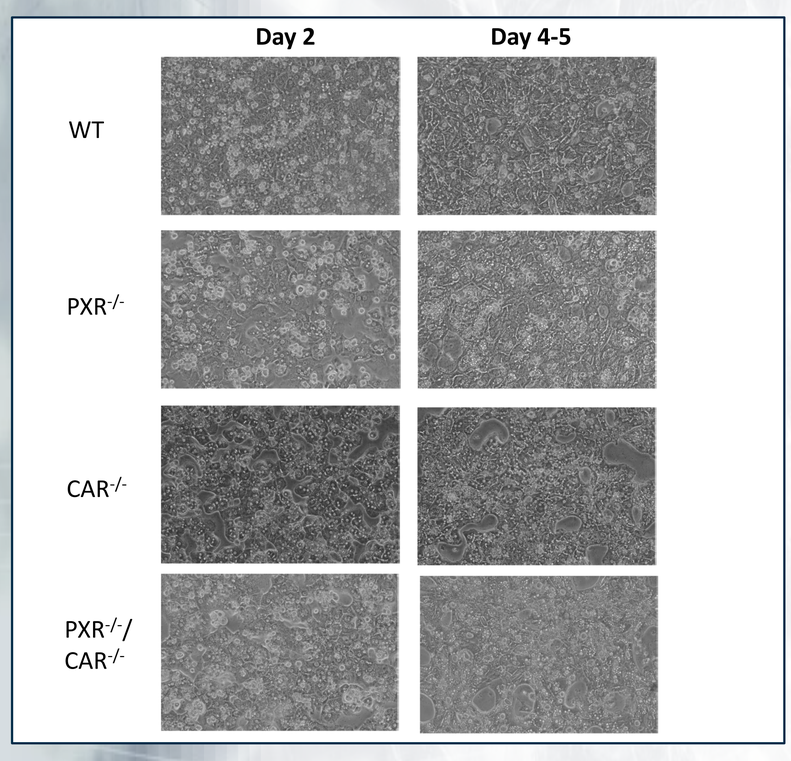
Confirmation of Functional PXR and CAR knock-out.
Cryopreserved hepatocytes were cultured on collagen coated plates for 4 days. On day 2, culture medium was replaced with medium supplemented with PCN 10 µM or TCPOBOP 250nM and changed again on day 3 (48-hr treatment). Hepatocytes pooled then RNA isolated and purified. Each compound was dissolved in 20% DMSO/RHIM before adding to the RHIM for culturing. The rough concentration of DMSO in the medium is 0.08%, and that concentration of RHIM/DMSO is used as our vehicle/control treatment.

Quantitative RT-PCR. Hepatocytes homogenized in TRIzol Reagent (Life Technologies, USA) with ceramic beads using a Precellys homogenizer. Total RNA was purified from homogenate and 100µg of total RNA was DNAse-I treated and purified (RNA Clean-up) using an RNeasy Mini-kit (Qiagen, USA). First strand cDNA was synthesized from 400ng to 1µg of purified RNA using the RT^2 First Strand Kit from Qiagen RT^2 Profiler PCR Array System. First strand cDNA was used in Taqman assays (Table 1; ThermoFisher, USA) for Phase II enzymes and transporters as single-plex reactions following the manufacturer's recommended conditions on Bio-Rad's CFX96 Real-Time PCR Detection System. Average fold-change was calculated normalizing to three reference genes (ActB, Hprt1 and Gapdh).

Table 1. Gene List Taqman Assays

Gene Symbol	Alias	Refseq #	Official Full Name	Taqman Assay ID
Cyp3a23/3a1	-	NM_013105.2	cytochrome P450, family 3, subfamily a, polypeptide 23/polypeptide 1	Rn03062228_m1
Cyp3a18	-	NM_145782.1	cytochrome P450, family 3, subfamily a, polypeptide 18	Rn00595752_m1
Cyp2b2	-	NM_001198676.1	cytochrome P450, family 2, subfamily b, polypeptide 2	Rn02786833_m1
Cyp2c11	-	NM_019184.2	cytochrome P450, subfamily 2, polypeptide 11	Rn01502203_m1
Cyp1a1	-	NM_012540.2	cytochrome P450, family 1, subfamily a, polypeptide 1	Rn01418019_g1
Cyp1a2	-	NM_012541.3	cytochrome P450, family 1, subfamily a, polypeptide 2	Rn00561082_m1
Slco1a2	Oatp2	NM_131906.1	solute carrier organic anion transporter family, member 1A2	Rn00756233_m1
Alb		NM_134326.2	Albumin	Rn00592480_m1
Reference genes				
ActB	-	NM_031144.3	actin, beta	Rn00667869_m1
Hprt1	Hprt	NM_012583.2	hypoxanthine phosphoribosyltransferase 1	Rn01527840_m1
Gapdh	Gapd	NM_017008.4	glyceraldehyde-3-phosphate dehydrogenase	Rn01775763_g1

Results

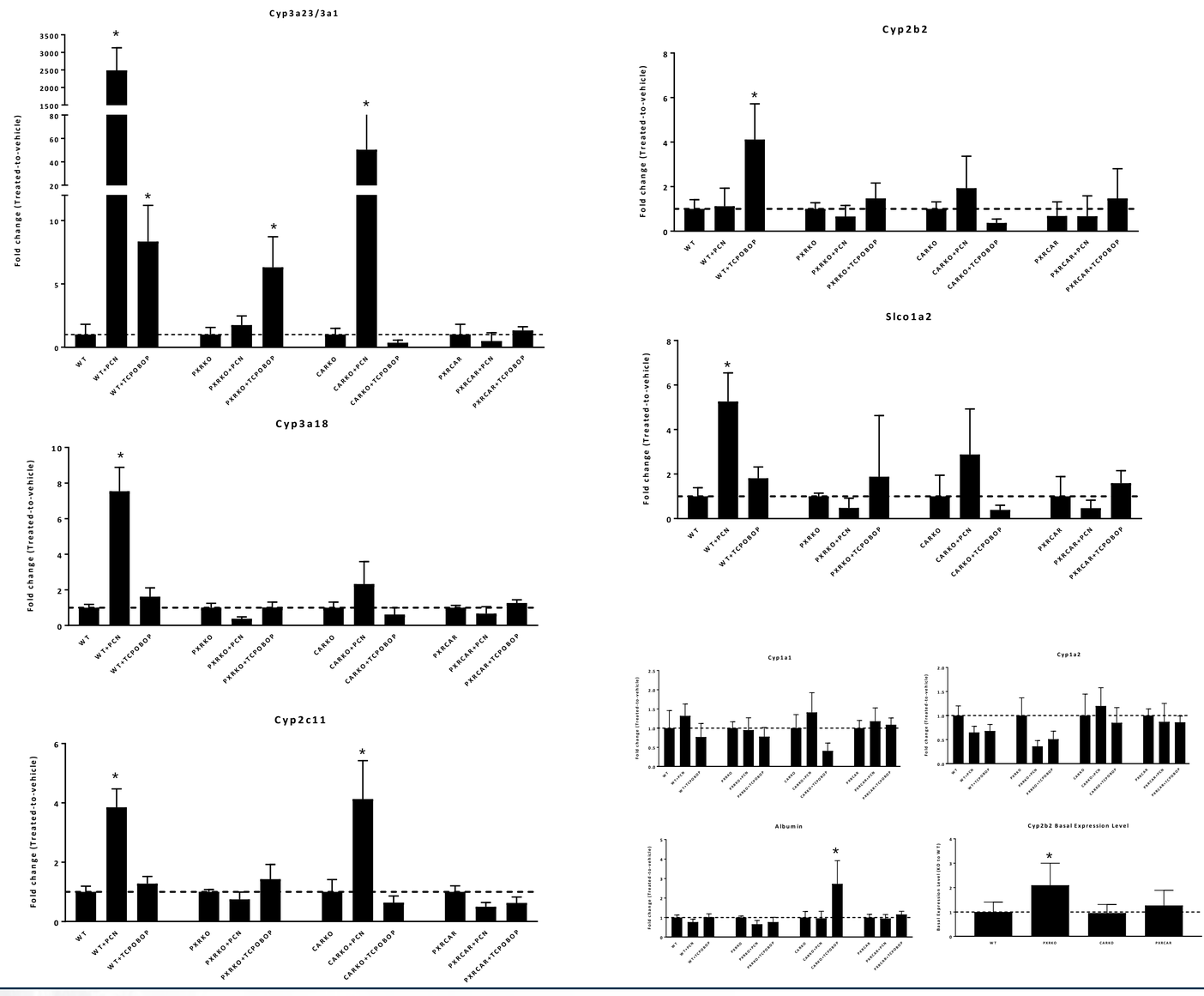


Monolayer Comments: Good attachment efficiency at 60-80 % and continues to develop a monolayer confluency of 75-90 % by day 3. Good morphology and remains intact for 5 days in culture. Photomicrographs, 100X, Phase contrast.

Drug Metabolizing Enzyme	Substrate (µM)	Incubation Time (minutes)	Metabolite Quantified	Activity (pmol/minute/million cells)			
				WT	*PXR-/-*	*CAR-/-*	*PXR-/-/CAR-/-*
ECOD	7-Ethoxycoumarin (100)	30	7-Hydroxycoumarin	827 ± 39	386 ± 115	106 ± 12	273 ± 34.7
UGT	7-Hydroxycoumarin (100)	30	7-Hydroxycoumarin glucuronide	922 ± 114	1311 ± 140	1298 ± 691	1660 ± 107
Sulfotransferase	7-Hydroxycoumarin (100)	30	7-Hydroxycoumarin sulfate	97 ± 4.3	188 ± 20.5	362 ± 52.7	311 ± 24.1

CYP450 Activity Assessment: The hepatocytes were incubated at a cell density of 0.5 million hepatocytes/mL in a 12-well plate (500,000 hepatocytes/well) for the designated time durations with isoform-selective substrates. The metabolites were identified and analyzed using LC-MS/MS.

Gene Expression Analysis (Select genes)



- ***Loss of PXR mediated PCN activation of Cyp3a23/3a1, 3a18, 2c11 and Slco1a2 (Oatp2);*** The loss of TCPOBOP induction of Cyp2b2 in the PXR KO is due to elevated Cyp2b2 basal expression levels when compared to WT (see bottom right graph)
- ***Loss of CAR mediated TCPOBOP activation of Cyp3a23/3a1 and Cyp2b2***

Values are fold change PCN or TCPOBOP to vehicle treated, +/- standard deviation.* = significantly different from vehicle treated, same genotype ($p<0.05$, t-test).

Conclusions and Future Directions

- Successful in the isolation and cryopreservation of rat hepatocytes from three knockout models: PXR, CAR and PXR/CAR
- Loss of PXR and CAR specific induction of P450 and transporter genes under compound treatment. Analogous to in vivo observations[1].
- These models, both in vivo[1] and the isolated hepatocytes, should be useful for studying metabolism of xenobiotic compounds and hepatotoxicity.
- These models are also critical components for the humanization of the cytochrome P450 pathways in the SD rat and subsequent isolated hepatocytes.

Acknowledgements

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References

1. Kevin P. Forbes Evguenia Kouranova, Daniel Tinker, Karen Janowski, Doug Cortner, Aaron McCoy and Xiaoxia Cui (2017) Creation and Preliminary Characterization of Pregnane X Receptor and Constitutive Androstane Receptor Knockout Rats. *Drug Metabolism and Disposition* 45:1068-1076.

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