Tissue Distribution, Ontogeny, and Chemical Induction of Aldo-Keto Reductases in Mice^S

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ABSTRACT

Aldo-keto reductases (Akr) are a conserved group of NADPH-dependent oxido-reductase enzymes. This study provides a comprehensive examination of the tissue distribution of the 16 substrate-metabolizing Akr in mice, their expression during development, and whether they are altered by chemicals that activate distinct transcriptional factor pathways. Akr1c6, 1c14, 1c20, and 1c22 are primarily present in liver; Akr1a4, 1c18, 1c21, and 7a5 in kidney; Akr1d1 in liver and kidney; Akr1b7 in small intestine; Akr1b3 and Akr1e1 in brain; Akr1b8 in testes; Akr1c14 in ovaries; and Akr1c12, 1c13, and 1c19 are expressed in numerous tissues. Liver expression of Akr1d1 and Akr1c is lowest during prenatal and postnatal development. However, by 20 days of age, liver Akr1d1 increases 120-fold, and Akr1c mRNAs increase as

much as 5-fold (Akr1c19) to 1000-fold (Akr1c6). Treatment of mice with chemical activators of transcription factors constitutive androgen receptor (CAR), pregnane X receptor (PXR), and the nuclear factor-erythroid-2 (Nrf2) transcription factor alters liver mRNAs of Akr. Specifically, CAR activation by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) increases mRNAs of Akr1b7, Akr1c6, Akr1c19, and Akr1d1, whereas PXR activation by 5-pregnenolone-16 α -carbonitrile (PCN) increases the mRNA of Akr1b7 and suppresses mRNAs of Akr1c13 and Akr1c20. The Nrf2 activator 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-lm) induces mRNAs of Akr1c6 and Akr1c19. Moreover, Nrf2-null and Nrf2 overexpressing mice demonstrate that this induction is Nrf2-dependent.

Introduction

Aldo-keto reductases (Akr) are a conserved group of NADPH-dependent oxido-reductase enzymes with (α/β)₈-barrel structural similarities (Jez et al., 1997). Most Akr are monomeric 34–37 kDa proteins; however, multimeric proteins are found in Akr2, Akr6, and Akr7 family members. Akr have been identified in multiple species, including eubacteria, fungi, plants, and vertebrates. Akr metabolize a wide variety of substrates, such as steroid hormones, carbohydrates, and xenobiotics (Seery et al., 1998). The substrate specificity is dependent on three flexible loops (A, B, and C) (Hoog et al., 1994).

Three Akr families have been identified. Mammalian Akr (Table 1) are found in the Akr1, Akr6, and Akr7 families (Jin and Penning, 2007). The Akr6 family members are involved in inactivation of voltage-gated potassium channels (Xie et al., 2011). The Akr7 family members reduce a metabolite of aflatoxin (B1-dyhydrodiol), a toxin produced by the fungus *Aspergillus flavus*, preventing the aflatoxin metabolite from forming damaging protein-adducts (Guengerich and Johnson, 1999).

Akr1 is the largest family of Akr. The Akr 1c, 1d, and 1e families are essential in the metabolism of hormones, including androgens, estrogens, and progesterone, as well as prostaglandins (Seery et al.,

1998; Penning et al., 2000). Akr1a4 is involved in the reduction of D-glyceraldehyde to glycerol. The Akr1b family of enzymes (Akr1b3, 1b7, and 1b8) is responsible for the reduction of toxic aldehydes generated during lipid peroxidation and steroidogenesis (Spite et al., 2007). In addition to detoxification activity, Akr1b3 and Akr1b7 also have prostaglandin F₂ α synthase activity (Kabututu et al., 2009).

The Akr1c subfamily has been viewed as a drug target because many Akr1c enzymes selectively metabolize steroid hormones as well as xenobiotics. In mice, nine members of the Akr1c family have been identified: Akr1c6, Akr1c12, Akr1c13, Akr1c14, Akr1c18, Akr1c19, Akr1c20, Akr1c21 and Akr1c22 (Deyashiki et al., 1995; Du et al., 2000; Matsumoto et al., 2006). Akr1c6 is required for the formation of testosterone from 4-androstenedione (Rheault et al., 1999). Akr1c12 and Akr1c13 oxidize alicyclic alcohols, aliphatic alcohols, 3 α -hydroxysteroids, 17 β -hydroxysteroids, and 20 α -hydroxysteroids (Endo et al., 2006, 2007). Akr1c18 inactivates progesterone by reducing it to its inactive metabolite, 20 α -hydroxyprogesterone (Mao et al., 1997). Akr1c20 and Akr1c21 metabolize multiple steroid hormones (Matsumoto et al., 2006; Dhagat et al., 2008).

Akr are important for the regulation of many endogenous pathways. Akr1c19 reduces isatin in the gastrointestinal tract. Isatin is a pharmacologically active molecule produced by intestinal bacteria (Ishikura et al., 2005). Akr1e1 reduces anhydro-D-fructose to anhydro-D-glucitol (Sakuma and Kubota, 2008), which is important in the regulation of glycogen. Akr are also important in the synthesis of bile acids from cholesterol (Stolz et al., 1984; Dufort et al., 1996). Akr1c6 is responsible for the 3 α -reduction, and Akr1d1 is responsible for 5 β -reduction of

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ABBREVIATIONS: AFB1, aflatoxin B1; AG, 1,5-anhydro-D-glucitol; AhR, aryl hydrocarbon receptor; Akr, aldo-keto reductase; CAR, constitutive androstane receptor; CDDO-lm, 2-cyano-3,12 dioxooleana-1,9 dien-28-imidazolide; HKO, Keap1 hepatocyte knockout; HSD, hydroxysteroid dehydrogenase; KD, Keap1-knockdown; Nrf2, nuclear factor-erythroid-2; PCN, 5-pregnenolone-16 α -carbonitrile; PCR, polymerase chain reaction; PPAR α , peroxisome proliferator-activated receptor- α ; PXR, pregnane X receptor; RPL13A, ribosomal protein L13; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene.

TABLE 1
Mouse Akrs

Akr	Activity	Substrates	Products	Reference
Akr1a4	Aldehyde reductase	D-Glucuronic acid D-Glucurono-7'-lactone	L-Gulonic acid L-Gulono-7'-lactone	(Takahashi et al., 2012)
Akr1b3	Aldose reductase	Prostaglandin H ₂	Prostaglandin F _{2α}	(Hyndman et al., 2003)
Akr1b7	Aldose reductase	Prostaglandin H ₂ 3-keto bile acids	Prostaglandin F _{2α} 3β-hydroxy bile acids	(Hyndman et al., 2003; Schmidt et al., 2011)
Akr1b8	Phospholipid reductase	POVPC POHyPC PONyPC	PHVPC PHHyPC PHNyPC	(Spite et al., 2007)
Akr1c6	HSD Testosterone synthesis	4-Androstenedione Dehydroepiandrosterone 5α-Androstane-3,17-dione androsterone	5-Androstene-3β,17α-diol epiT 5α-Androstane-17α-ol-3-one 5α-Androstane-3α,17α-diol	(Bellemare et al., 2005)
	Bile acid synthesis	5β-Cholestan-7α-ol-3-one	5β-Cholestan-3α,7α -diol	
Akr1c12	HSD	Alicyclic alcohols Aliphatic alcohols 3α-Hydroxysteroids 17β-Hydroxysteroids 20α-Hydroxysteroids	5β-Androstane-3α,17β-diol 4-Pregnene-17α,20α-diol-3-one Other products	(Endo et al., 2006)
Akr1c13	Oxidation of nonsteroidal alcohols, low HSD activity	Alicyclic alcohols Aliphatic alcohols 3α-Hydroxysteroids 17β-Hydroxysteroids 20α-Hydroxysteroids	Not identified	(Endo et al., 2007)
Akr1c14	Predicted HSD	Unknown	Unknown	(Ishikura et al., 2004)
Akr1c18	HSD	Progesterone	20α-Hydroxyprogesterone	(Mao et al., 1997)
Akr1c19	HSD	Isatin	3-Hydroxy-2-oxoindole	(Usami et al., 2001; Ishikura et al., 2005)
Akr1c20	HSD	α-Dicarbonyl compounds 4-Androsten-3α-ol-17-one 5α-Pregnan-3α,21-diol-20-one	Not identified	(Matsumoto et al., 2006)
Akr1c21	HSD	5β-Androstan-17-ol-3-one 5β-Androstan-17β-ol-3-one	Not identified	(Dhagat et al., 2008)
Akr1c22	Dihydrodiol dehydrogenase	Not identified	Not identified	NCBI Accession# BAD02825
Akr1e1	Anhydro-D-fructose reductase	Anhydro-D-fructose	Anhydro-D-glucitol	(Sakuma and Kubota, 2008)
Akr1d1	Δ ⁴ -3-Ketosteroid-5β-reductase, bile acid synthesis	4-Cholesten-7α-ol-3-one	5β-Cholestan-7α-ol-3-one	(Kondo et al., 1994)
Akr6a4	Shaker channel β-subunit (Kvb2)	1-Palmitoyl-2-arachidonoyl-3-phosphatidyl choline	Not identified	(Xie et al., 2011)
Akr6a8	Shaker channel β-subunit (Kvb1)	Not identified	Not identified	(Pan et al., 2008)
Akr6a14	Shaker channel β-subunit (Kvb3)	Not identified	Not identified	NCBI Accession# NP_034729
Akr7a5	Aflatoxin aldehyde reductase	Aflatoxin	Aflatoxin B ₁ -dialdehyde	(Hinshelwood et al., 2002)

HSD, hydroxysteroid dehydrogenase; NCBI, National Center for Biotechnology Information.

4-cholesten-7α-ol-3-one in the production of bile acids (Penning et al., 2000; Mindnich et al., 2011).

Akrs are involved in the activation of polycyclic aromatic hydrocarbons (Penning et al., 1996). Polycyclic aromatic hydrocarbons are byproducts of fossil fuel combustion and tobacco smoke. Human Akrs 1B1 and 1B10 catalyze the oxidation of benzopyrene-7,8-diol to form a ketol. Tautomerization of this product leads to 7,8-dihydroxybenzopyrene. After several auto-oxidation steps, an *o*-quinone is formed, benzopyrene-7,8-dione. Benzopyrene-7,8-dione subsequently forms adducts to DNA, resulting in mutations (Penning et al., 1996, 1999).

Akrs also detoxify fungal toxins. Aflatoxin B1 (AFB1) is a mutagenic mycotoxin that is a contaminant in food. AFB1 spontaneously hydrolyzes to form AFB1-dialdehyde. AFB1-dialdehyde is cytotoxic because of its ability to form protein adducts. Akr7a (aflatoxin aldehyde reductase) reduces AFB1-dialdehyde to form an alcohol, and this reduction prevents AFB1-dialdehyde from forming lysine adducts (Ellis et al., 1993).

In general, little is known about the regulation of Akrs. Therefore, in the present study, the tissue distribution of mouse Akrs was determined in adult male mice. In addition, an ontogeny study was performed to determine the pattern of Akr expression during liver and kidney development. Lastly, we sought to determine whether chemicals known to induce drug-metabolizing enzymes and transporters will also alter the mRNA expression of various Akr isoforms. The inducers include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which activates the aryl hydrocarbon receptor (AhR); 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), which activates the constitutive androstane receptor (CAR); 5-pregnenolone-16α-carbonitrile (PCN), which activates the pregnane X receptor (PXR); clofibrate, which activates the peroxisome proliferator-activated receptor (PPARα); and 2-cyano-3,12 dioxooleana-1,9 dien-28-imidazolide (CDDO-Im), which activates nuclear factor-erythroid-2 (Nrf2). Finally, those *Akr* genes with expression altered by the Nrf2 agonist CDDO-Im, were further examined in genetically altered mice that have either hepatic knockout or overexpression of Nrf2.

Materials and Methods

Chemicals. TCPOBOP and 5-pregnenolone-16a-carbonitrile (PCN) were purchased from Sigma-Aldrich (St. Louis, MO). TCDD was a gift from Dr. Karl Rozman (University of Kansas Medical Center, Kansas City, KS). Clofibrate was purchased from Fisher Scientific (Hampton, NH). (CDDO-Im) was a gift from Reata Pharmaceuticals (Irving, TX).

Mice. Eight-week old C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) to determine the tissue distribution, ontogeny, and chemical induction of Akrs. Nrf2-null mice (Chan et al., 1996) were obtained from Dr. Jefferson Chan (University of California, Irvine, CA). Keap1-knockdown (KD) mice (Okada et al., 2008) were supplied by Dr. Masayuki Yamamoto (Tohoku University, Sendai, Japan). Keap1-hepatocyte knockout (HKO) mice were engineered as described previously (Wu et al., 2011). Nrf2-null and Keap1-KD mice were backcrossed into the C57BL/6 background, and 99% congenicity was confirmed by Jackson Laboratory. The mice were housed on corn cob bedding according to the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All animals were given ad libitum access to water and standard rodent chow (Harlan Teklad 8604; Harlan Teklad, Madison, WI).

For tissue distribution studies, 8-week-old male ($n = 6$) and female ($n = 6$) mice were used. Liver, kidney, lung, stomach, duodenum, jejunum, ileum, colon, brain, testes, ovaries, and heart were collected from mice. The tissues were snap-frozen in liquid nitrogen. The intestine was longitudinally dissected, rinsed in saline, and divided into three equal-length sections (referred to as duodenum, jejunum, and ileum) before being frozen in liquid nitrogen. All tissues were stored at -80°C . For the ontogeny study, liver was collected from male mice ($n = 5$) at postnatal days $-2, 0, 1, 3, 5, 10, 20$, and 45 . Kidney was collected from male mice ($n = 4$) at postnatal days $-2, 0, 10, 20$, and 30 . Brain was collected from male mice ($n = 4$) at postnatal days $0, 10, 20$, and 30 . For chemical induction of Akrs by Cyp inducers, the activators were administered to male mice ($n = 5$) for 4 days (intraperitoneally) as described previously (Cheng et al., 2005). Tissues were collected 24 hours after the final dose.

RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction. Total RNA was isolated from 50 mg of snap-frozen liver using RNAzol B reagent purchased from Tel-Test Inc. (Friendswood, TX), and quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at an UV absorbance of 260 nm. Two micrograms of total RNA was used for cDNA synthesis performed using a high-capacity cDNA synthesis kit from Applied Biosystems (Foster City, CA). Oligonucleotide primer sequences were designed by NCBI Primer-BLAST to be specific to 16 Akkr mRNAs (detailed in Table 2). Primers were then aligned to the mouse genome to test for specificity. Real-time polymerase chain reaction (PCR) analysis was performed using a StepOnePlus instrument from Applied Biosystems. The mRNA of each gene was adjusted to the mRNA of RLP13. Relative levels of each gene were evaluated using the $\Delta\Delta\text{Ct}$ method.

Statistics. Statistical differences between multiple groups were analyzed by a one-way analysis of variance followed by Duncan's post hoc test. Statistical significance was considered at $P < 0.05$.

Results

Tissue Distribution of Mouse Akrs. The mRNAs of sixteen Akrs were quantified in 12 mouse tissues as shown in Fig. 1. Akkr6 members were not quantified because they are only distantly related (15%–30% homology) to other Akrs, and they function as inactivators of voltage-gated potassium channels (McCormack and McCormack, 1994; Barski et al., 2009). Tissues from six male and six female mice were used to determine the expression of these Akkr genes in liver, kidney, lung, stomach, duodenum, jejunum, ileum, colon, brain, testes, ovaries, and heart. No gender differences in expression of any Akkr mRNA were detected (unpublished data). Akkr1a4 mRNA was highest in kidney; however, there was also expression in liver, lungs, stomach, and small intestine. Akkr1b3 mRNA was 20 times more prevalent in brain than in other tissues (Fig. 1). Akkr1b7 mRNA was highest in the small intestine, with similar amounts in duodenum, jejunum, and ileum (Fig. 1). There was also quantifiable Akkr1b7 mRNA in kidney; however, it was only 1/20 of that in small intestine. Akkr1b8 mRNA was highest in testes and second highest in stomach (Fig. 1). Akkr1c6 mRNA was found to be highest in liver, with levels at least 200-fold higher than those quantified in other tissues (Fig. 1). Akkr1c12 and Akkr1c13 were expressed in liver, stomach, duodenum, jejunum, ileum, and colon (Fig. 1). Akkr1c14 was expressed at levels at least 4-fold higher in liver and ovaries than in other tissues (Fig. 1). Akkr1c18 was expressed in kidneys at levels 500-fold higher than those in other tissues (Fig. 1). Akkr1c19 mRNA was expressed in liver, kidney, stomach, duodenum, jejunum, ileum, and colon (Fig. 1). Akkr1c20 was mainly expressed in liver, at quantities 50-fold higher than seen in other tissues (Fig. 1). Akkr1c21 was expressed mainly in kidney. Akkr1c22 was expressed highest in liver and stomach, with the mRNA about 20-fold higher than in other tissues (Fig. 1). Akkr1d1 was expressed highest in liver and kidney, with the mRNA about 66- and 33-fold higher, respectively, than in other tissues (Fig. 1). Akkr1e1 mRNA was highest in brain, with a value 3-fold higher than in other tissues (Fig. 1). Akkr7a5 mRNA was 10-fold higher in kidney than in other tissues (Fig. 1).

Ontogeny of Akrs in Mouse Liver, Kidney, and Brain. A developmental study was performed to determine the pattern of Akkr expression during liver, kidney, and brain development. First, the Akrs

TABLE 2
Oligonucleotide primer sequences designed to be specific to 16 Akkr mRNAs

Gene	Forward	Reverse
Akr1a4	ATAGCCCCTTGGGTTCTCT	GATCTGAGCTGGAGATCGGC
Akr1b3	GGGCTATTATAAGGTGCGCGC	TGGGCATCTTGGTGCCGTTGTT
Akr1b7	GGAACGTGAGGGCCTGTGACCT	ACGGATCTCATCAAGCAAGTGGACC
Akr1b8	CCACGTTCTGTGAACCTAGTAC	CGTTGCAATAGGCATACGCGCA
Akr1c6	TGCTCCAGAACTCGTACGGGT	TCCCGGCTTCATGGCCATTGG
Akr1c12	GCAGAGTTCCTTGCTGACCACCC	CCAGCATCTGAGTCACATCACCAGC
Akr1c13	ACCACCCAGAGTATCCATTGTGGA	TGGTCATATTGCCAGCATCACTGTC
Akr1c14	TCTCAAGACCTGCGTGGTTGCA	GGTTCCAAACCCAGTGCAGGG
Akr1c18	CTTCTAGGTTCAGAGCAGTGGCTGAG	TGTCATAGGTGCCAAGCCAGG
Akr1c19	ATGCACCTGCTCCATTGGAGAG	GCTGTGCGTAGAAGTCATGACACA
Akr1c20	GAACGCCCTGCGCTGATTGCC	ACCCAAAGCAACTCATCACCTGCA
Akr1c21	CGAAGATCGTGTAGGACAGGC	TTGCAGTGACCGTACCAAGG
Akr1c22	GGCCACTTAATTCCTGCCCT	GTGACGAACAGGTCTTCTCTCT
Akr1d1	TAACCAAGGTGGAGTGCCACCCG	CCATGATGGGTTGCGGCAGGT
Akr1e1	AGCCTGGTTTGAGGGTCAGGC	GAGCCACCGAGGGGACGGTA
Akr7a5	TACTGCGACGGCCAGTCCGA	CCCTTCCAGGGGTTGGCCT

Fig. 1. Tissue distribution of mouse *Akr* genes. Total RNA from 12 tissues (liver, kidney, lung, stomach, duodenum, jejunum, ileum, colon, brain, testes, ovaries, and heart) in male and female C57BL/6 mice ($n = 12$) was analyzed by real-time PCR. Gene control used was ribosomal protein L13 (RPL13a). Data are presented as mean $\pm$ S.E.M.

that were significantly expressed in livers of adult mice were examined, namely *Akr1c6*, *Akr1c14*, *Akr1c19*, *Akr1c20*, *Akr1d1*, *Akr1e1*, and *Akr7a5*. The mRNAs of these Akrs were quantified at postnatal days -2 , 0 , 1 , 3 , 5 , 10 , 20 , and 45 .

Akr1c6 mRNA increased between 5 and 10 days of age and reached adult expression by 20 days of age (Fig. 2). *Akr1c14* mRNA increased markedly between 10 and 20 days of age (Fig. 2). *Akr1c19* and *Akr1c20* mRNA also increased between 10 and 20 days of age. *Akr1c19* mRNA increased 5-fold, and *Akr1c20* increased more than 2-fold during this time interval (Fig. 2). *Akr1e1* mRNA also increased between 10 and 20 days of age, which was about a 4-fold increase (Fig. 2). *Akr1d1* mRNA was barely detectable before birth (-2), was detectable at day 1 of age, and reached adult levels at 20 days of age (Fig. 2). The mRNA of *Akr7a5* did not change markedly in livers from 2 days before birth to 45 days of age.

The Akrs that were significantly expressed in kidney of adult mice were examined to determine how expression changes during development. The mRNAs were quantified at postnatal days -2 , 0 , 10 , 20 , and 30 . The mRNAs of *Akr1a4* and *Akr1c19* in kidneys did not change during the first month of life (Fig. 3). *Akr1c18* and *Akr1c21* mRNAs in kidneys of mice increased 7-fold and 200-fold, respectively, from 2 days before birth to 30 days of age (Fig. 3). *Akr1d1* and *Akr7a5* mRNA both increased about 3-fold from 2 days before birth to 30 days of age (Fig. 3).

The Akrs that were expressed in brain of adult mice were also examined to determine whether the mRNA of the Akrs change during the first month of life. The mRNAs of *Akr1b3*, *Akr1c18*, *Akr1e1*, and

Akr7a5 were quantified from brains of mice at postnatal days 0, 10, 20, and 30. There were no significant age-relevant changes in the mRNAs observed for any of these Akrs (Supplemental Fig. 1).

Chemical Induction of Mouse Akrs. Five different chemicals known to activate various transcription factors that induce cytochrome P450 enzymes as well as other drug metabolizing enzymes and transporters were used in this study (Aleksunes and Klaassen, 2012). The inducers used were: TCDD for AhR, TCPOBOP for CAR, PCN for PXR, clofibrate for PPAR α , and CDDO-Im for Nrf2. *Akr1a4*, *Akr1b3*, *Akr1b8*, *Akr1c21*, *Akr1e1*, and *Akr7a5* had low amounts of measurable mRNA in liver before and after inducer treatment (Supplemental Fig. 2). *Akr1b7* mRNA was very low in liver of control mice but increased about 300-fold after TCPOBOP and 15-fold after PCN administration (Fig. 4).

Akr1c6 mRNA increased about 50% after TCPOBOP and 100% after CDDO-Im administration (Fig. 4). *Akr1c12* mRNA was unresponsive to any of the activators. *Akr1c13* mRNA decreased more than 50% after PCN and clofibrate treatments (Fig. 4). *Akr1c14* was unresponsive to all five activators (Fig. 4). *Akr1c18* mRNA increased over 400% after clofibrate administration. *Akr1c19* mRNA increased more than 3-fold after TCPOBOP and more than 5-fold after CDDO-Im administration. *Akr1c20* mRNA decreased 80% after TCPOBOP and 75% after treatment with PCN (Fig. 4). *Akr1c22* mRNA decreased 80% after TCPOBOP and TCDD administration, and was undetectable after PCN administration. *Akr1d1* mRNA more than doubled after TCPOBOP administration (Fig. 4).

Fig. 2. Ontogeny of mouse *Akr* genes in liver. Total RNA from mice at each age ($n = 5$) was analyzed by real-time PCR. Gene control used was RPL13a. Data are presented as mean $\pm$ S.E.M.

Gene Dose-Response of Nrf2 on *Akr1c6* and *Akr1c14*. Because CDDO-Im is a Nrf2 activator and increases the expression of *Akr1c6* and *Akr1c14*, a gene dose-response model was employed to further analyze the role of Nrf2 in the regulation of these genes. Nrf2 is a short-lived protein that is readily ubiquitinated by CUL3-RBX1 and degraded by the 26S proteasome. The ubiquitylation of Nrf2 depends on Kelch-like ECH-associated protein 1 (Keap1), which functions as a substrate adaptor for CUL3-RBX1 (Kobayashi et al., 2004). If Keap1 is not present or inactivated, Nrf2 translocates into the nucleus. The four types of mice used to make the *Nrf2* gene dose response were Nrf2-null, wild-type, Keap1-KD, and Keap1-HKO. In this model, Nrf2-null mice possess no Nrf2 activity, and the Keap1-HKO mice possess the highest level of Nrf2, as characterized previously (Wu

et al., 2011). Mice that lack Nrf2 had a 50% decrease in *Akr1c6* mRNA (Fig. 5). The Keap1-HKO mice had a 5-fold increase in *Akr1c19* (Fig. 5). The transcription of a gene whose transcription was not affected by CDDO-Im treatment was also examined (not shown). The elimination of Nrf2 or Keap1 had no effect on the transcription of *Akr1c14*, which mirrored the results from the chemical treatment study. These results further indicate that Nrf2 is important for the regulation of *Akr1c6* and *Akr1c19*.

Discussion

*Akr*s are important enzymes for the synthesis of endogenous compounds as well as for the detoxification of xenobiotics. To better

Fig. 3. Ontogeny of mouse *Akr* genes in kidney. Total RNA from mice at each age ($n = 4$) was analyzed by real-time PCR. Gene control used was RPL13a.

Fig. 4. Effects of chemical induction on C57BL/6 mouse Akkr mRNA expression in liver. The dose of chemical treatment ($n = 5$) was described under *Materials and Methods*. Data are presented as mean $\pm$ S.E.M. Asterisks (*) indicate statistically significant increase/decrease in mRNA level after treatment compared with the control group by analysis of variance ($P < 0.05$).

understand the expression of these genes, this study first quantitatively determined the expression of the Akrs in various tissues, although other studies have performed limited tissue distribution studies of a few Akrs. The present study examined more Akrs, more mice, and contained mice of both.

The Akkr1b family (Akkr1b3, 1b7, and 1b8) of enzymes is responsible for the reduction of toxic aldehydes generated during lipid peroxidation and steroidogenesis (Spite et al., 2007). Akkr1b family members (Akkr1b3, Akkr1b7, and Akkr1b8) also possess prostaglandin $F_{2\alpha}$ synthase activity (Kabutu et al., 2009). In addition, Akkr1b7 is important for metabolizing 3-keto bile acids to 3 β -hydroxy bile acids. Toxic bile acids, such as desoxycorticosterone acetate, are converted to less toxic 3 β bile acids, such as 3 β DCA, by Akkr1b7 (Schmidt et al.,

2011). The present data indicates for the first time that Akkr1b3 is expressed mainly in brain. Akkr1b7 was expressed mainly in intestine, which confirms an earlier published result that was obtained using the RNase protection assay (Lau et al., 1995). In the present study, Akkr1b8 was located mainly in testes and stomach. This finding somewhat contradicts a previous report using qualitative reverse transcriptase PCR of Akkr1b8 in which expression was observed in equal amounts in most tissues except brain and kidney, which exhibited lower expression (Salabei et al., 2011). The results of the present study may be different because the previous study did not include stomach and was only determined after 28 cycles of PCR.

The Akkr1c family members (Akkr1c6, 1c12, 1c13, 1c14, 1c18, 1c19, 1c20) are viewed as drug targets because of their roles in steroid

Fig. 5. Analysis of mRNA expression of Akkr1c6, and Akkr1c19 by real-time PCR analysis of the four groups ($n = 5$ per group). The different genotypes included Nrf2-null, wild-type, Keap1-KD, and Keap1-HKO. Data are presented as mean $\pm$ S.E.M. Asterisks (*) indicate statistically significant differences from wild-type mice ($P < 0.05$).

biogenesis and xenobiotic metabolism. Previous reports have demonstrated that many of these Akr1c family members are expressed in livers of mice (Vergnes et al., 2003; Velica et al., 2009). Akr1c6 is almost exclusively expressed in liver (Fig. 1), which corresponds with previously published data (Vergnes et al., 2003; Velica et al., 2009). Akr1c12 and Akr1c13 mRNA are ubiquitously expressed. Akr1c14 expression is highest in liver and in ovaries. This finding was different from a previous report that found the highest expression of Akr1c14 in kidney, and similar expression in liver, kidney, stomach, intestine, colon, lung, and ovary (Velica et al., 2009). Akr1c18 mRNA was found mainly in the kidney and at a lower concentration in brain. This result also differed from the publication of Velica et al., which reported expression only in ovary. These differences could be the result of differences in mouse strain because Velica et al. used CD1 mice, whereas this study used C57BL/6 mice. Another possible cause of the variance is that Velica et al. used pregnant and nonpregnant female mice. Pregnancy might have increased the expression of Akr1c18 mRNA.

The expression of *Akr* genes was examined at different stages of liver, kidney, and brain development. The liver undergoes marked developmental changes after birth. Liver changes from a hematopoietic organ before birth to an organ necessary for xenobiotic metabolism after birth. The expression of the P450 enzymes and transporters in liver changes over the course of development (Hart et al., 2009; Cui et al., 2012a,b; Lu et al., 2012). An increase in the mRNA of several *Akr1c* genes in liver was observed after birth (Fig. 2). Akr1d1 mRNA increased over 100-fold between birth and day 45. Akr1c20 mRNA increased over 50-fold, Akr1c6 increased over 10-fold, and Akr1c4 and Akr1c19 increased in liver to a lesser extent between birth and day 45 (Fig. 2).

The expression of several *Akr* genes in kidney was also quantified in kidney at multiple ages. Similar to some of the *Akr* genes described previously in liver, two *Akr* mRNAs increase during development in kidney. Akr1c18 mRNA increased over 7-fold, and Akr1c21 mRNA increased in kidney about 200-fold from birth to 30 days of age (Fig. 3).

Various transcription pathways are known to increase various drug-metabolizing enzymes in liver, therefore we sought to determine whether these pathways also alter the expression of Akrs. AhR activation led to the increase of mRNA of Akr1b7. CAR activation led to increased mRNA of Akr1b7, which had been described previously (Liu et al., 2009), and Akr1c6. PXR activation caused an increase of Akr1b7 and a decrease in the mRNAs of Akr1c13, Akr1c20, and Akr1c22 (Fig. 4). PXR activation has been reported previously to cause a repression of Sult1e1 by decreasing the HNF4 α interaction with the Sult1e1 promoter (Kodama et al., 2011). This might also be the mechanism by which PXR causes a decrease in Akr1c18, 1c20, and 1c22. Activation of PPAR α led to an increase of the mRNAs of Akr1b7 and Akr1c18. PPAR α activation also led to a decrease in mRNA of Akr1c13. Activation of Nrf2 resulted in the upregulation of Akr1c6 and Akr1c19 (Fig. 4). These results were further confirmed by knockout of the *Nrf2* and *Keap1* genes (Fig. 5).

Akr1d1 is expressed mainly in liver and kidney (Fig. 1). This discovery that Akr1d1 is found in liver is not surprising because Akr1d1 was previously described as serving a role in bile acid biogenesis (Gonzales et al., 2004). Akr1d1 mRNA, similar to many drug-metabolizing genes, increases in liver from birth to adulthood (Fig. 2). Akr1d1 mRNA was increased 3-fold by CAR activation (Fig. 4). The change in expression of Akr1d1 may contribute to changes in bile acid profile in the developing organism.

Akr1e1 is responsible for the reduction of 1,5-andyro-D-fructose (AF), which is produced from glucose, to 1,5-anhydro-D-glucitol (AG). The physiologic role that AG plays in glucose metabolism is not

well understood, but patients with diabetes mellitus have elevated levels of AG in blood and urine (Sakuma and Kubota, 2008). Akr1e1 was found mainly in brain (3-fold higher than other tissues) in the present study (Fig. 1), which is different from Vergnes et al. who reported expression in every tissue. They performed reverse transcriptase PCR, using between 28 and 35 cycles and only presenting the gel. The signal from some of these tissues might be out of the logarithmic range. The present study used a fluorescent system and quantified the signals at 21 cycles, which was within the logarithmic range for all of the tissues. Akr1e1 mRNA increased in liver from birth to adulthood (Fig. 2).

In conclusion, the present study examined the regulation of the mRNAs of Akrs in tissues, the ontogeny of *Akr* mRNAs in mouse liver, and whether activators alter their expression. This study demonstrated that mouse *Akr* mRNAs have developmental patterns similar to those of multiple drug-metabolizing enzymes; that is, the mRNA of Akrs 1c6, 1c14, 1c19, 1c20, 1d1, and 1e1 increased, starting at birth and continuing to increase until adulthood. This study also demonstrates that AHR, CAR, PXR, PPAR α , and Nrf2 transcriptional pathways influence the transcription of some *Akr* genes.

Authorship Contributions

Participated in research design: Pratt-Hyatt and Klaassen

Conducted experiments: Pratt-Hyatt and Lickteig

Contributed new reagents: Lickteig

Performed data analysis: Pratt-Hyatt

Wrote or contributed to the writing of the manuscript: Pratt-Hyatt and Klaassen

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