Tissue Expression, Ontogeny, and Inducibility of Rat Organic Anion Transporting Polypeptide 4

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ABSTRACT
To date, organic anion transporting polypeptide 4 (Oatp4; Slc21a10) is known as a liver-specific and sodium-independent transporter that mediates transport of a variety of compounds. The purpose of this study was to determine whether Oatp4 mRNA expression is specific to the liver compared with Oatp1, 2, 3, and 5. In addition, the effect of gender and age was determined by assessing the expression of Oatp4 mRNA during the postnatal development of rats. Furthermore, to determine whether Oatp4 gene expression is coordinately modulated by drug-metabolizing enzyme inducers, male rats were administered chemicals known to induce the expression of drug-metabolizing enzymes through six mechanisms: the aryl hydrocarbon receptor, constitutive androstane receptor, pregnane X receptor, peroxisome proliferator-activated receptor, electrophile response element, or CYP2E1 inducers. The levels of Oatp1, 2, 3, 4, and 5 mRNA were measured using the branched DNA signal amplification technique. The tissue distribution of Oatp4 was almost exclusively expressed in liver in contrast to Oatp1, 2, 3, and 5. The hepatic expression of Oatp4 was low in newborn rats and increased gradually to the adult level with no significant difference between genders. The expression of Oatp4 was not consistently induced by any of the six groups of enzyme inducers. These findings continue to suggest that Oatp4 is expressed specifically in the liver. The preference of Oatp4 for endogenous compounds coupled with its refractory response to known drug-metabolizing enzyme inducers suggests that Oatp4 may be largely responsible for the homeostasis of endogenous rather than exogenous chemicals, including pharmaceuticals.

Hepatic biotransformation and/or biliary excretion mechanisms are important for the detoxification of endogenous and exogenous compounds. For some compounds to be eliminated from blood into bile, the coordinate expression of transporters on the sinusoidal membrane of the hepatocyte, the phase I and II drug-metabolizing enzymes, and transporters on the canalicular membrane for excretion are all necessary. It has been demonstrated that hepatic uptake can be extremely efficient, for example, some solutes are almost completely removed from the blood upon a single passage through the liver, which may be due to specialized transporters expressed on the sinusoidal membrane. The Oatp family of transporters mediates transport of a variety of substrates, including organic anions, cations, and neutral compounds in a Na+-independent manner. Among the Oatps that have been cloned in rats (Oatp1, 2, 3, 4, and 5), the one that is most specifically and predominantly expressed in liver will probably be critical for the disposition of various compounds through liver.

Newborn rats are susceptible to the toxicity of a number of xenobiotics (Klaassen, 1973), such as cardiac glycosides, which is due to the decreased capacity of newborns to transport xenobiotics into the liver (Klaassen, 1972; Stacey and Klaassen, 1979). Therefore, in contrast to adults, low expression of a specific transporter that removes chemicals from blood in the newborn may result in more of the xenobiotic avoiding the first-pass removal by the liver and instead reaching other organs to cause injury.

It is also known that certain drug-metabolizing enzyme inducers, such as phenobarbital and pregnenolone-16α-carbonitrile, are intrinsically able to increase the hepatic uptake of a number of compounds (Klaassen and Plaa, 1968; Klaassen, 1970, 1974). Therefore, it appears that the coordinate regulation between phase I and II drug-metabolizing enzymes and hepatic uptake transporters in the liver may occur in response to chemical stimuli. Current knowledge of nuclear ligand-activated receptor regulation of phase I and phase II enzymes suggests that these transcription factors may play a role in coordinately regulating phase I and phase II enzymes, as well as transporter protein (Rausch-Derra et al., 2001).

ABBREVIATIONS: Oatp, organic anion transporting polypeptide gene family; PCN, pregnenolone-16α-carbonitrile; SD, Sprague-Dawley; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; CAR, constitutive androstane receptor; PXR, pregnane X receptor; PPAR, peroxisome proliferator-activated receptor; EpRE, electrophile response element; bDNA, branched DNA; QR, quinone reductase; kb, kilobase.

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Therefore, the aims of this study were to 1) determine whether Oatp4 is specifically expressed in rat liver compared with the other Oatps, 2) determine whether there is a gender difference in its expression, 3) determine its postnatal expression, and 4) determine whether it is coordinately regulated by inducers of phase I and II drug-metabolizing enzymes.

Materials and Methods

Tissue Distribution. Male and female Sprague-Dawley (SD) rats (200–250 g) (n = 5/gender) were purchased from Sasco (Wilmington, MA), and housed according to the American Animal Association Laboratory Animal Care guidance. Each rat was euthanized in a CO₂ atmosphere and decapitated. The tissues were dissected from each rat and snap-frozen in liquid nitrogen. The intestine was longitudinally dissected and rinsed in saline, and intestinal epithelial cells were mechanically scraped from the intestine and snap-frozen in liquid nitrogen. All the tissues were stored at −80°C.

Ontogeny. Rats were bred in the animal facilities at University of Kansas Medical Center (Kansas City, KS), and livers from male and female SD rats were collected at 0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 days of age (n = 5/gender/age).

Induction by Microsomal Enzyme Inducers. Male SD rats (n = 5/treatment) were treated with the following classes of chemicals, doses, and routes of administration: aryl hydrocarbon receptor ligands: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (3.9 μg/kg i.p. in corn oil, 1 day), indole-3-carbinol (56 mg/kg p.o. in corn oil), β-naphthoflavone (100 mg/kg i.p. in corn oil), and 3,3’,4,4’,5-pentachlorobiphenyl (40 μg/kg i.p. in corn oil); constitutive androstane receptor (CAR) ligands: phenobarbital (80 mg/kg i.p. in saline), 2,2’,4,4’,5-pentachlorobiphenyl (16 mg/kg p.o. in corn oil), and diallyl sulfide (500 mg/kg i.p. in corn oil); pregnane X receptor (PXR) ligands: PCN (50 mg/kg i.p. in corn oil), spironolactone (75 mg/kg i.p. in corn oil), and dexamethasone (50 mg/kg i.p. in corn oil); peroxisome proliferator-activated receptor (PPAR) ligands: clofibric acid (200 mg/kg i.p. in saline), diethylhexylphthalate (1200 mg/kg p.o. in corn oil), and perfluorodecanoic acid (40 mg/kg i.p. in corn oil, 1 day); electrophile response element (EpRE) activators: ethoxyquin (150 mg/kg p.o. in corn oil) and oltipraz (150 mg/kg p.o. in corn oil); and CYP2E1 inducers: isoniazid (200 mg/kg i.p. in saline), acetylsalicylic acid (500 mg/kg p.o. in corn oil), and streptozotocin (100 mg/kg i.p. in sodium citrate, 1 day). Control rats were administered corn oil i.p. and corn oil p.o., and saline i.p. All animals were treated for 4 days unless otherwise noted, and injection volumes were 5 ml/kg. Livers were collected on day 5, snap-frozen in liquid nitrogen, and stored at −80°C.

RNA Isolation. Total RNA was isolated using RNazol B reagent (Tel Test B, Friendswood, TX) per the manufacturer’s protocol. The concentration of total RNA in each sample was quantified spectro-photometrically at 260 nm. The integrity of each RNA sample was evaluated by formaldehyde–agarose gel electrophoresis before analysis.

Branched DNA (bDNA) Signal Amplification Assay. The mRNA for Oatp1, Oatp2, Oatp3, Oatp4, and Oatp5 was measured using the bDNA assay (Quantigene bDNA signal amplification kit; Bayer Diagnostics, East Walpole, MA), with modifications according to Hartley and Klaassen (2000). The gene sequences of rat Oatp 3, 4, and 5 as well as quinone reductase were accessed from GenBank (Table 1). Multiple oligonucleotide probe sets (containing capture probes, label probes, and blocker probes) specific to a single mRNA transcript were designed using ProbeDesigner software, version 1.0 (Table 1). Every probe developed in ProbeDesigner was submitted to the National Center for Biotechnological Information for nucleotide comparison by the basic logarithmic alignment search tool (BLASTs; NCBI, Bethesda, MD) to ensure minimal cross-reactivity with other known rat sequences and expressed sequence tags. Oligonucleotides with a high degree of similarity (>80%) to other rat gene transcripts were eliminated from the design. The probe sets for CYP1A1, CYP2B1/2, CYP3A1/23, CYP2E1, and GAPDH were previously described by Hartley and Klaassen (2000), as well as the probe sets for Oatp1 and Oatp2 (Rausch-Derra et al., 2001). Probes were designed with a melting temperature of approximately 63°C, enabling hybridization conditions to be held constant (i.e., 53°C) during each hybridization step, and for each probe set. All probes were synthesized (i.e., 50-nmol synthesis scale) by Operon Technologies (Palo Alto, CA), and obtained desalted and lyophilized. Total RNA (1 μg/μl) was added to each well (10 μl/well) of a 96-well plate containing 100 μl of capture hybridization buffer and 100 μl of each diluted probe set. For each gene, total RNA was allowed to hybridize to the probe set overnight at 53°C. Subsequent hybridization steps were carried out as per the manufacturer’s protocol, and luminescence was measured with a Quantiplex 320 bDNA luminometer interfaced with Quanti-plex data management software, version 5.02, for analysis of luminescence from 96-well plates. The luminescence for each well is reported as relative light units per 10 μg of total RNA.

Statistics. Differences between control and chemical-treated rats as well as gender were analyzed by analysis of variance, followed by Duncan’s multiple range analysis. Statistical significance was considered at p < 0.05.

Results

Tissue Distribution of Rat Oatps. To investigate the tissue distribution of rat Oatps, total RNA pooled from five male or five female of the following tissues was examined for each gene: liver, kidney, lung, stomach, duodenum, jejunum, ileum, large intestine, cerebellum, cerebral cortex, heart, spleen, lymph node, thymus, adrenal, pancreas, spinal cord, muscle, skin, testes, ventral prostate, dorsal prostate, ovary, uterus, mammary, tongue, placenta, eye, olfactory bulb, hippocampus, frontal cortex, caudate, brain stem, thalamus, pituitary, blood vessel, nasal epithelium, and thyroid (data not shown). Finally, 10 major tissues were selected to quantify Oatp1, 2, 3, 4, and 5 mRNA levels and are shown in the first five figures. Expression of Oatp1 mRNA (Fig. 1) was highest in male kidney, high in liver of both male and female rats, and minimal in the other eight tissues. No gender difference in Oatp1 mRNA expression was apparent in liver. However, much higher expression of Oatp1 mRNA was detected in male kidney than in female kidney. Expression of Oatp2 mRNA (Fig. 2) is higher in liver and brain tissues, such as cerebellum and cerebral cortex, than in the other tissues. There was no gender difference in Oatp2 expression in the 10 tissues examined. Expression of Oatp3 mRNA (Fig. 3) was highest in lung, moderate in brain tissues, such as cerebellum and female cerebral cortex, and low in the other tissues. The expression of Oatp3 is significantly higher in female lung and cerebral cortex than that in male rats. There is no gender difference in Oatp3 expression in the other eight tissues. Expression of Oatp4 mRNA (Fig. 4) was highest in liver with minimal expression in the other nine tissues. There was no gender difference in Oatp4 expression. Expression of Oatp5 mRNA (Fig. 5) was almost exclusively expressed in kidney with minimal expression in other tissues. No gender difference in Oatp5 expression was apparent.

Ontogeny of Oatp4. Oatp4 is liver-specific as indicated in Fig. 4, so its ontogenic expression was investigated using liver. Expression of Oatp4 in rats of various ages from day 0 to 45 is shown in Fig. 6. No differences in expression were
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observed in male and female rats at any age, and thus were combined. The expression of Oatp4 was present, but low before day 15, and increased approximately 5-fold by day 45. Additional evaluation of Oatp4 expression at embryonic day 18 demonstrated that this transporter is actually present before birth (data not shown).

**Induction of Oatp4.** To validate that each of the chemicals selected induced the expected cognate nuclear receptor-activated response, CYP1A1, CYP2B1/2, CYP3A1/23, CYP4A2/3, QR, and CYP2E1 mRNA levels were determined in response to ligands known to activate the aryl hydrocarbon, CAR, PXR, PPAR, EpRE, and CYP2E1 pathways, respectively. As shown in Fig. 7, three of four selected aryl hydrocarbon receptor ligands (TCDD, \( \beta \)-naphthoflavone, and PCB126) increased the expression of CYP1A1 mRNA by more than 5-fold (Fig. 7, panel 2). The PXR ligands (PCN, spironolactone, and dexamethasone) all increased the mRNA of CYP3A1/23 more than 12-fold (Fig. 7, panel 3). The PPAR ligands (clofibric acid, diethylhexylphthalate, and perfluorodecanoic acid) all increased the mRNA of CYP4A2/3 more than 6-fold (Fig. 7, panel 4). The EpRE activators, namely, ethoxyquin and oltipraz, both increased the mRNA of quinone reductase at least 4-fold (Fig. 7, panel 5). The CYP2E1 inducers (isoniazid, acetylsalicylic acid, and streptozotocin) all increased the mRNA of CYP2E1 at least 3-fold (Fig. 7, panel 6). Therefore, CYP1A1, CYP2B1/2, CYP3A1/23, CYP4A2/3, QR, and CYP2E1 were all significantly induced in livers of rats treated with chemicals known to induce gene expression of these enzymes, indicating that appropriate doses and times were used.

However, in contrast to the marked induction of the drug-metabolizing enzymes as shown in Fig. 8, the change in Oatp4 mRNA was not consistently increased by any class of drug-metabolizing enzyme inducer. Only ethoxyquin signifi-

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**TABLE 1**

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a Target region refers to the sequence of the mRNA transcript as enumerated in the GenBank file.

b Function relates to the function of the oligonucleotide probe in the Quantigene assay.

c CE, capture probe; LE, label probe; BL, blocker probe.
cantly increased Oatp4 mRNA by about 53%, and PCN increased Oatp4 by 40%, but the latter was not statistically significant. In summary, none of these chemical classes consistently increased Oatp4 mRNA expression, which was in stark contrast to the induction observed for the phase I drug-metabolizing enzymes.

**Discussion**

Rat Oatp4 was first cloned by Kakyo et al. (1999). However, this was later shown to be a splice variant of the complete Oatp4. Recently, the full length of Oatp4 was cloned in two independent studies (Cattori et al., 2000; Choudhuri et
Because Oatp4 was concluded to be a liver-specific protein after examining only a few tissues using Northern blot analysis, it was decided to further determine the expression pattern of Oatp4 at the mRNA level. To do this, a wide range of tissues was assessed using the bDNA signal amplification assay. The bDNA assay is the application of the well-developed enzyme-linked immunosorbent assay format on the mRNA analysis and directly measures signal from the transcript itself. Moreover, the judicious design of capture and label extender probes by ProbeDesigner software and database screening in National Center for Biotechnology Information make this methodology highly sensitive and specific.

**Fig. 3.** Tissue distribution of Oatp3 mRNA. Total RNA from both male and female (n = 5/gender) SD rat tissues was analyzed by the bDNA assay for expression of Oatp3. Data are presented as mean ± S.E.M. *, statistically significant difference between male and female rats.

**Fig. 4.** Tissue distribution of Oatp4 mRNA. Total RNA from both male and female (n = 5/gender) SD rat tissues was analyzed by the bDNA assay for expression of Oatp4. Data are presented as mean ± S.E.M.
This approach is advantageous because it uses the specificity inherent with short oligonucleotide probes and the sensitivity of long cDNA probes. The bDNA system has been demonstrated that it is comparable to quantitative reverse transcription-polymerase chain reaction with regard to sensitivity (Collins et al., 1997; Guenthner and Hart, 1998).

The Oatp family of transporter proteins is expressed in various tissues. In the present study, Oatp1 was expressed in liver and its highest expression was detected in male kidney. A previous report by Jacquemin et al. (1994) indicated that a strong positive hybridization signal was observed in liver and kidney, and a weak hybridization signal in brain, lung, skeletal muscle, and proximal colon when washing was performed at 58°C in the Northern blot analysis. But when more stringent conditions were used (65°C), signals were only detected in liver and kidney (Jacquemin et al., 1994). However,
they did not indicate the strain and gender of rats used. Higher Oatp1 expression was observed in the kidney of male than female rats (Lu et al., 1996). Lu and colleagues indicated that testosterone administration to female rats produced a tendency toward higher Oatp1 expression, but the difference was not statistically significant. They interpreted their finding that the kidney may have tissue-specific transcription factors that respond to testosterone, whereas these factors are not present in liver. The present study shows that the male rat kidney has the highest amount of Oatp1 mRNA, whereas the female kidney lacks abundant levels of Oatp1 mRNA. Interestingly, the livers of both male and female rats express high levels of Oatp1 mRNA. The present findings also suggest that Oatp1 expression is largely limited to the liver and kidney in rats.

A previous study examined the tissue distribution of Oatp2 using Northern blot analysis (Noé et al., 1997). However, two different lengths of transcripts were detected. The larger transcript (~4 kb) was detected strongly in liver, moderately in brain, and weakly in kidney. The smaller transcript (2.8 kb) of Oatp2, which was considered to be a shorter untranslated region of the Oatp2 gene at the 3’ end, was strong in kidney and liver, but weak in brain. The present study using specific probes of Oatp2 indicates its expression is mainly in liver and brain (Fig. 2).

The tissue distribution of Oatp3 was first reported by Abe...
et al. (1998) using Northern blot analysis with a 900-base pair probe. They found two different length transcripts, 3.9 and 2.8 kb. The 3.9-kb transcript was detected in kidney and liver, and the 2.8-kb transcript in kidney and retina. As pointed out by Walters et al. (2000), the 900-base pair probe used by Abe et al. (1998) encompassed a 260-nucleotide coding region that is highly conserved among the Oatp family members. Using an RNase protection assay, which selected a 3’-untranslated region sequence (nucleotides 2288–2404) of the Oatp3 gene, Walters et al. (2000) found Oatp3 mRNA was present in lung, brain, and retina. In the same manuscript, using reverse transcription-polymerase chain reaction, Oatp3 was also detected in the small intestine and liver. The present study used an Oatp3-specific probe set designed from both 3’- and 5’-untranslated regions (sequence information provided by H. Waters and P. Dawson, Wake Forest, Winston-Salem, NC; and T. Abe, Tohoku University School of Medicine, Sendai, Japan) with the branched DNA amplification assay, and indicated that lung has the highest amount of Oatp3 followed by the brain. In the previous studies only male rats were used and the present study indicates that Oatp3 is more highly expressed in females than males (Fig. 3).

Previous studies indicated that Oatp4 is liver-specific using Northern blot analysis with a limited number of tissues (Kakyo et al., 1999; Choudhuri et al., 2000). The present study used 38 tissues to support previous findings that Oatp4 is relatively specifically expressed in liver (Fig. 4). It is also of interest to note that the relative light units for Oatp4 mRNA in the liver of rats is much higher than that of the other Oatps, suggesting that Oatp4 may also be the Oatp in the greatest abundance in the liver.

The tissue distribution of rat Oatp5 mRNA has not been investigated thus far. The present study indicates that it is relatively kidney-specific (Fig. 5), which is consistent with the mouse ortholog (Choudhuri et al., 2001).

Therefore, the data demonstrate that only Oatp4 mRNA is liver-specific, and in addition may be the highest expressed Oatp in liver. Moreover, Oatp4 appears to be expressed exclusively in liver of mice (Ogura et al., 2000), rats (Kakyo et al., 1999; Choudhuri et al., 2000), and humans (Abe et al., 1999).

The substrate specificity of Oatp4 suggests that Oatp4 may play an important role in the hepatic uptake of a variety of compounds (Cattori et al., 2000). In addition, the high level of expression of Oatp4 may contribute significantly to the hepatic first-pass effect, which contributes to the rapid clearance of orally administered drugs, and is a major concern for the development of drugs that need to bypass the presystemic elimination and reach other organs to elicit a pharmacological effect. Moreover, the specificity of Oatp4 for the liver may produce liver-selective toxicity by certain xenobiotics, which are selectively transported by Oatp4 and accumulate in the liver.

Oatp4 is not only important in the disposition of xenobiotics but also in formation of bile and bile flow. Most bile acids are taken up by the liver from the portal vein blood by uptake transporters on the sinusoidal membrane (e.g., Oatp4), subsequently excreted across the canalicular membrane, and ultimately distributed to the small intestine to complete enterohepatic cycle. Therefore, understanding the regulation of Oatp4 may also facilitate our understanding of cholestasis.

In the present study, it was shown that Oatp4 mRNA undergoes an increase in expression in rat liver during the first few weeks of life. The delayed expression of Oatp4 during the perinatal period may result in the increase in susceptibility of newborn rats to xenobiotic-elicited toxicity, immaturity of bile formation, and physiological neonatal cholestasis. LD50 of ouabain in newborns is dramatically decreased compared with that in adult rats (Klaassen, 1972). Whether low expression of Oatp4 is responsible for susceptibility of newborns to ouabain or other chemicals has not been examined. It was reported that Oatp1 and Ntcp undergo ontogenic regulation in rat liver (Hardikar et al., 1995; Dubuisson et al., 1996). Therefore, Oatp1 and Ntcp might also contribute to the immaturity of bile formation. However, Ntcp mainly mediates transport of bile acids and Oatp1 does not readily transport ouabain (Bossuyt et al., 1996; Noé et al., 1997; Eckhardt et al., 1999).

For some compounds, hepatic uptake by transporters is required to provide substrates to the drug-metabolizing enzymes in the liver. Therefore, it is reasonable to hypothesize that there could be coordinate regulation between transporters and the phase I and II drug-metabolizing enzymes. Thus, cooperative regulation of phase I and II and transport could protect an organism from xenobiotic-induced toxicity. In fact, nuclear ligand-activated receptors that regulate phase I and II drug-metabolizing enzymes have been demonstrated to coordinate regulation of the influx transporter Oatp2, but not Oatp1 (Hagenbuch et al., 1999; Rausch-Derra et al., 2001). However, whether these nuclear ligand-activated receptors also enhance the expression of Oatp4 had not been investigated. Even though the doses and time regimens for the various ligands for the nuclear receptors to induce the cytochrome P450 drug-metabolizing enzymes were confirmed in the present study, none of the six classes of inducers consistently increased Oatp4. This indicates that Oatp4 does not appear to be coordinately regulated with these phase I and II drug-metabolizing enzymes.

The fact that Oatp4 is refractory to these known ligands of nuclear receptors suggests that Oatp4 may be largely responsible for the homeostasis of endogenous compounds (Cattori et al., 2000), which is consistent with the results of its functional studies. Therefore, Oatp4 might mainly contribute to the formation of bile flow and removal of endogenous compounds. In addition, because constitutive expression of Oatp4 is high, it might be capable of eliminating exogenous compounds even though it is not inducible. The full range of substrates of Oatp4 is not known, especially the xenobiotics. The data of full range of substrates of Oatp4 will provide insight into the physiological and pathological functions of Oatp4.

In conclusion, only Oatp4 is liver-specific and is the most highly expressed among the known rat Oatps. Thus, this transporter may play the most important role in the selective uptake of xenobiotics by the liver. There is no gender deference in the expression of Oatp4. Expression of Oatp4 is low in newborn rats, and increases with age to adult levels. Therefore, susceptibility of newborn animals to certain xenobiotic-elicited toxicities might be due to their low expression of Oatp4. And finally, expression of Oatp4 mRNA is not readily regulated by any known class of phase I and II drug-metabolizing enzyme inducers. The expression of Oatp4 was only measured at mRNA levels in the present study. Further
studies are required to determine whether protein levels parallel the mRNA levels of Oatp4.

References


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