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Differential Gene Expression of Organic Anion Transporters in Male and Female Rats

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Sex-related differential gene expression of organic anion transporters (rOAT1, rOAT2, and rOAT3) in rat brain, liver, and kidney was investigated. There were no sex differences in the expression of rOAT1 mRNA. rOAT2 mRNA was abundant in the liver and weakly expressed in the kidney of male rats; however, the OAT2 gene was strongly expressed in both organs of females. The abundance of rOAT2 mRNA markedly increased in castrated male rat kidney; however, treatment of castrated male rats with testosterone led to a decrease of rOAT2 mRNA. Expression of rOAT3 mRNA in intact female rats was found in the kidney and brain, whereas in males rOAT3 mRNA was also found in the liver. rOAT3 mRNA markedly decreased in the liver of castrated male rats but increased in testosterone-treated castrated male rats. Moreover, rOAT3 mRNA increased in the hypophysectomized female rat liver, indicating that rOAT3 is an inducible isoform. The present findings suggest that sex steroids play an important role in the expression and maintenance of OAT2/3 isoforms in the rat liver and kidney. Our results provide information on the differential gene expression of OAT isoforms with sex hormone dependency.

Key Words: **organic anion transporter; sex differences; organic anion; pharmacokinetics.**

The carrier-mediated movement of solutes across membrane plays a central role in the maintenance of both cellular and organismic homeostasis. An understanding of the physiological function of transporters is important because of the close relationship between transport and the detoxification and elimination of a wide range of drugs, environmental contaminants and endogenous substrates. Generally, lipophilic substrates are actively metabolized by various cytochrome P450 isoforms (CYPs) in the liver and kidney cells of all vertebrate species and converted into more water-soluble substrate (1). In the kidney, these water-soluble substrates are actively secreted from the body by transport across the basolateral and/or luminal membranes (2–9). Likewise, one of the major functions of the liver is the clearance of a large variety of endogenous and exogenous substances including organic anions and cations by transport across the sinusoidal membrane into the hepatocytes (6–8). Thus, the detoxification and the elimination of endogenous and exogenous substances from the body seem to be determined by transporters and CYPs.

Recently, we have isolated organic anion transporter 1 (rOAT1) from a rat kidney cDNA library (10). OAT1 homologs have been subsequently cloned and characterized by our group and other investigators (11–19). However, the tissue distribution differs between isoforms. For example, rOAT1 and rOAT3 mRNAs are predominantly expressed in the kidney (10, 17), whereas rOAT2 (NLT) abundantly exists in the liver (15, 16).

In turn, sex differences in the disposition of many drugs and chemicals have been well accepted (20, 21). Such a sexual dimorphism becomes normally apparent at puberty and is associated with lower rates of metabolism in females compared to males toward the biotransformation of many substrates. For instance, liver microsomes from females catalyze certain reactions more efficiently than males (20, 21). This differentiation may exist because of the sex-dependent expression of CYP isoforms in the liver and/or the kidney (1, 20, 22, 23). In this respect, Kamataki *et al.* and Imaoka *et*

Abbreviations used: OAT, organic anion transporter; CYP, cytochrome P450.

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al. have reported that liver-specific CYP2C11/12 and kidney-specific CYP4A2 isoforms are expressed in a sex-dependent manner, and the expression levels are regulated by androgen or by the secretion pattern of the growth hormone (22–24). Based on all of these findings, the expression of OAT isoforms in the liver and kidney may also be regulated by sex steroids since there are many reports concerning sex differences in pharmacokinetics of drugs (25–28). Furthermore, the elucidation of sex differences in the distribution of OAT isoforms would be important for the prediction of drug pharmacokinetics and unexpected side effects. In this article, we describe the differential gene expression of rOAT1, rOAT2, and rOAT3 in male and female rats.

MATERIALS AND METHODS

Animals and treatment. Wistar male and female rats (7 weeks of age) and hypophysectomized male and female rats were used in this study. The rats were obtained from Nippon Zairyo Ctr. (Tokyo, Japan) after surgical operation and were fed a commercially available diet (MF, Oriental Yeast Co., Tokyo Japan) and water *ad libitum*. Castration surgery was done according to the method of Kamataki *et al.* (22). Some of the castrated male rats were surgically operated at day 42 after birth and were left 10 days with free access to food and water, then given a subcutaneous injection of testosterone propionate (in corn oil, 1 ml/kg) at a dose of 5 mg/kg, and of estradiol (in corn oil, 1 ml/kg) at a dose of 0.5 mg/kg, respectively, once a day for 10 consecutive days. Control rats were injected subcutaneously with the vehicle alone. Some of the hypophysectomized rats were surgically operated at day 49 after birth and were left 10 days with free access to food and water. All animals were kept in a light-, temperature-, and moisture-controlled room in wire-bottomed cages.

Tissue preparation. For total RNA isolation, all rats were killed by decapitation and the brain, liver, and kidney were immediately flash-frozen in liquid nitrogen. The tissues were stored at −80°C until use.

Total RNA isolation. Total RNA was isolated from the liver using the acid GTC–phenol–chloroform extraction method according to the method of Chomczynski and Sacchi (29). The liver was homogenized in a GTC solution [4.0 M guanidine thiocyanate containing 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl and 0.1 M 2-mercaptoethanol: 10 ml GTC solution/100 mg of liver tissue]. The RNA was extracted twice with phenol and chloroform (1 vol phenol/0.2 vol chloroform/1 vol GTC solution) and precipitated with isopropanol at room temperature. The pellet was dissolved in a 0.3 ml GTC solution and precipitated with isopropanol at −20°C. The resulting RNA pellet was washed with ice-cold 80% ethanol and dissolved in an appropriate volume of diethylpyrocarbonate-treated water. The RNA yield, purity and integrity were determined by the 260/280 nm absorbance ratio (>1.8) and checked with 1.0% agarose/formamide gel.

Northern blot analysis. Twenty micrograms of total RNA was denatured in 1× Mops (0.02 M Mops, pH 7.0, containing 0.3 M sodium acetate, and 1 mM EDTA), 2.2 M formaldehyde and 50% deionized formamide at 65°C for 10 min and then quick chilled on ice before loading onto a 1.0% agarose/formamide gel. RNA samples were stained with ethidium bromide (0.8 μg/ml), and loading efficiency was checked by ultraviolet. After electrophoresis, RNA was transferred onto a nylon membrane (Hybond N+, Amersham–Pharmacia Biotech, Buckinghamshire, England) for 18 h with 20× SSPE (3 M NaCl, 173 mM $NaH_2PO_4 \cdot 12\ H_2O$, 25 mM EDTA). Transferred RNA was fixed by 50 mM NaOH, and the membrane was probed with ^{32}P-labeled full-length rOAT1 (10), rOAT2 (16), and rOAT3 cDNA (17). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) using the sense primer 5′-TCCACCACCCTGTTGCTGTA-3′ and antisense primer 5′-ACCAGAGTCCATGCCATCAC-3′ yielding a 0.45-kb fragment. The RT-PCR was done according to the manufacturer's instructions (Toyobo, Osaka, Japan). For hybridization, the insert and RT-PCR product were labeled by random primer extension according to the method of Feinberg and Vogelstein (30) using a random primer labeling kit (^{T7}QuickPrime kit, Amersham–Pharmacia Biotech, Piscataway, NJ). The filter was prehybridized for 2 h at 42°C in a prehybridization solution (0.5 M sodium phosphate (pH 6.5), 50% formamide, 5× SSC, 5× Denhardt's, 280 μg/ml sheared denatured salmon sperm DNA). The filters were then incubated overnight at 42°C in the same solution containing about 5×10^6 cpm/ml ^{32}P-labeled cDNA probe. After hybridization, the membrane was washed with 2× SSC/0.1% SDS at 42°C for 30 min and with 0.1× SSC/0.1% SDS at 42°C for 30 min.

RESULTS AND DISCUSSION

Thus far, at least five different OAT isoforms (OAT1-5) are known to exist in mammals (10–19, 31, 32). OAT1 and OAT3 are strongly expressed in the kidney (10, 17), whereas OAT2 is predominantly found in the liver (15, 16). OAT4 is detected in the placenta, which is a female endocrine organ, in addition to the kidney (18). Thus, the expression level of individual OAT isoforms depends on the organ in which they are found. On the other hand, gender-related pharmacokinetic differences of many drugs and chemicals have been elucidated (25–28, 33, 34). Such sex-dependent alterations in pharmacokinetics may be related to sex differences in the expression of xenobiotic transporters, such as OAT, in the liver and kidney, because these transporters mediate the uptake of various kinds of drugs and chemicals. To test this hypothesis, we examined whether there exists a sex-related differential gene expression of OAT isoforms (rOAT1-3) in the brain, liver and kidney by Northern blot analysis.

First, we investigated whether there were sex differences in the expression of rOAT1, rOAT2, and rOAT3 mRNA in the brain, liver, and kidney. As shown in Fig. 1, we observed that rOAT1 mRNA is predominantly expressed in the kidney of male rats. There was no hybridization signal of rOAT1 mRNA in the liver and brain. The results obtained in female rats under similar experimental conditions are also shown in Fig. 1. Similar to the males, rOAT1 mRNA was highly expressed in the kidney of females. No hybridization signal was detected with rOAT1 mRNA in the female rat liver and brain even after long exposure. Therefore, we concluded that there is no sex-dependent differential gene expression of the rOAT1 in rats. Subsequently, we examined whether a gender-related differential gene expression of rOAT2 mRNA exists. Interestingly, there was a sex-differentiated expression of rOAT2 mRNA in the kidney, with a stronger signal in females than males. As shown in Fig. 1, rOAT2 mRNA was strongly detected in the liver of both

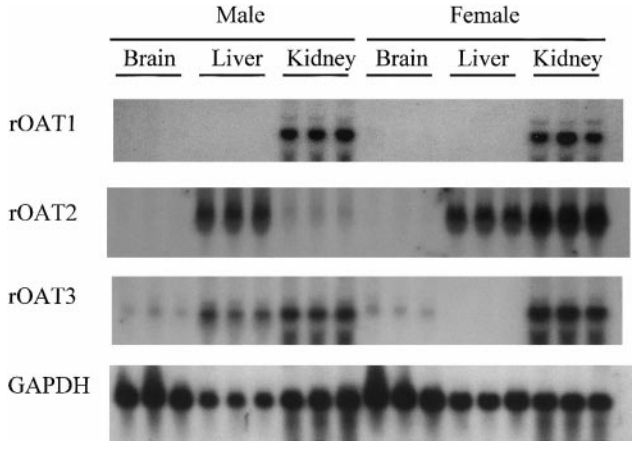

FIG. 1. Northern blot analyses of rOAT1, rOAT2 and rOAT3 mRNA in the brain, liver, and kidney of male and female rats. For each lane, 20 μg of total RNA isolated from various rat tissues was loaded onto 1.0% agarose/formamide gel and transferred to a nylon membrane with 20× SSPE. Total RNA samples obtained from three rats was loaded onto the agarose gel. The membrane was then hybridized with each ^{32}P-labeled full-length OAT isoform and GAPDH cDNA. Highly expressed 2.4-kb (rOAT1, kidney), 2.0-kb (rOAT2, liver and kidney), and 2.7-kb (rOAT3, brain, liver, and/or kidney) transcripts were detected under high-stringency conditions. Each panel shows a 3- to 4-day exposure. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Other experimental conditions and methods are described under Materials and Methods.

sexes, but we did not observe significant sex differences in the expression (Fig. 1). No positive signals for rOAT2 mRNA was detected in the brain of either sex. These results indicate that there is gender-related differential gene expression of rOAT2 mRNA in the kidney. The sex-dependent expression of rOAT3 mRNA is also represented in Fig. 1. The levels of rOAT3 mRNA in the brain, liver, and kidney of male rats exhibited a rank order of the kidney > liver > brain. Surprisingly, no rOAT3 mRNA was detected in the female rat liver even after long exposure (Fig. 1). The expression levels of rOAT3 mRNA in the brain and kidney of both sexes were similar. These results indicate that rOAT3 might be classified as a "male liver-specific" organic anion transporter rather than a "kidney-type" transporter. The results of this experiment also suggest that sex steroids play a key role in the expression of the rOAT2/3 isoform in rat liver and kidney.

To examine whether sex steroids mediate a sex-differentiated rOAT2 gene expression in the rat kidney (Fig. 1), we used castrated male rats for subsequent experiments. As shown in Fig. 2A, the expression of rOAT2 mRNA in the kidney increased significantly after castration. The finding suggests that the expression of this isoform would be mediated by sex hormone(s), such as testosterone. To test this hypothesis, we examined the effect of testosterone on the expression of rOAT2 mRNA. Castrated male rats were treated with testosterone at a dose of 5 mg/kg for 10 days. As expected, the expression of rOAT2 mRNA in the kidney markedly decreased in testosterone-treated male rats to levels similar to those in intact male rats. However, the expression of rOAT2 mRNA in the estradiol-treated castrated male rat kidney also decreased.

It is generally accepted that secretion patterns of growth hormones are regulated by the pituitary gland and that secretion of testosterone is regulated by growth hormones (22–24). Therefore, mRNA levels in the hypophysectomized female rat kidney were measured. As shown in Fig. 2B, the expression of rOAT2 mRNA in the hypophysectomized female rat kidney was markedly decreased. The result suggests that the expression of rOAT2 mRNA in the kidney is regulated by the pituitary-gonadal axis. Furthermore, our results also suggest that the expression of rOAT2 mRNA would be mediated through the presence of testosterone. However, it is not clear how this hormone is involved in the expression of the rOAT2 gene in the kidney. Further studies are needed.

Many sex differences in pharmacokinetics have been reported from *in vivo* studies for a number of clinically used drugs (25–28). For example, Ho and colleagues have found that men have a significantly higher clearance of salicylate than women (26). We have reported that [^{14}C]salicylate is a good substrate for rOAT2 (16). The results of our current studies (Fig. 1) together with our previous paper (16) suggest that the OAT2 isoform would be one of the key transporters influencing sex differences in salicylate pharmacokinetics in humans.

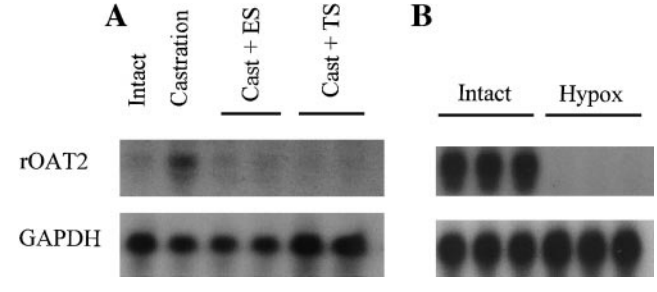

FIG. 2. Northern blot analyses of rOAT2 mRNA in the kidney of intact, castrated, and hypophysectomized rats. (A) Twenty micrograms of total RNA was isolated from the intact and castrated male rat kidney and loaded onto 1.0% agarose/formamide gel. Total RNA obtained from two intact and two castrated male rats were pooled, respectively, and individual total RNA was hybridized in the case of castrated male rats treated with sex steroids. A single 2.0-kb transcript was significantly detected in castrated males and weakly expressed in the intact and castrated rat kidney treated with sex steroids. (B) Hypophysectomized female rats were surgically operated at day 49 after birth and were left 10 days with free access to food and water. Cast, castrated male rats; ES, estradiol (0.5 mg/kg/day for 10 consecutive days); TS, testosterone (5.0 mg/kg/day for 10 consecutive days); Hypox, hypophysectomized male rats; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Other experimental conditions and methods are identical to those described in the legend to Fig. 1.

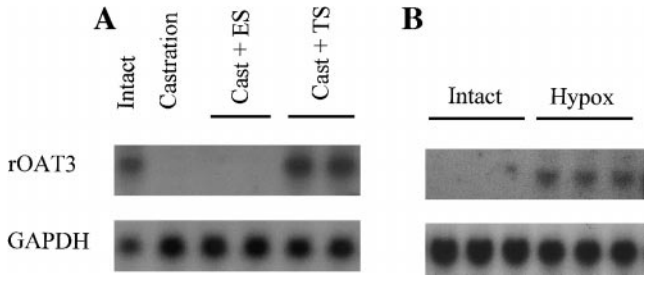

FIG. 3. Northern blot analyses of rOAT3 mRNA in the liver of intact, castrated, and hypophysectomized rats. (A) Twenty micrograms of total RNA isolated from the intact and castrated rat liver was loaded onto 1.0% agarose/formamide gel. Total RNA obtained from two intact rat and two castrated male rats were pooled, respectively, and individual total RNA was hybridized in the case of castrated male rats treated with sex steroids. A single 2.7-kb transcript was detected in the liver of intact male and castrated male rats treated with testosterone. No positive signal was detected in the liver of castrated male and castrated male rats treated with estradiol. (B) Twenty micrograms of total RNA isolated from the intact and hypophysectomized rat liver was loaded onto 1.0% agarose/formamide gel. A single 2.7-kb transcript was detected in the liver of hypophysectomized female rats. No positive signal was detected in the liver of intact female rats. Hypox, hypophysectomized female rats; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Other experimental conditions and methods are identical to those described in the legends to Figs. 1 and 2.

However, Montgomery and colleagues have reported that there are no sex-dependent differences in salicylate pharmacokinetics (33). Further studies will be required to resolve this apparent discrepancy.

The possible relationship between the rOAT3 mRNA expression and sex steroids in the castrated rat liver was next investigated. As shown in Fig. 3A, the levels of the expression of rOAT3 mRNA in the male rat liver decreased after castration as observed in the intact female rat liver. However, treatment of castrated rats with testosterone resulted in the recovery of rOAT3 mRNA to the intact male levels. Thus, the expression of rOAT3 mRNA in the liver seems to require the presence of testosterone. Estradiol treatment was also unable to restore the expression of rOAT3 mRNA in the castrated male rat liver (Fig. 3A). These findings suggest that the expression rOAT3 mRNA in the liver seems to require a presence of either endogenous or exogenous sex steroids. Interestingly, rOAT3 mRNA clearly increased in the hypophysectomized female rat liver (Fig. 3B). The result suggests that a OAT3 homolog(s) would be an inducible isoform although the molecular mechanisms in the induction of this isoform remain to be determined.

The uptake of certain drugs and chemicals from the blood into liver cells is regulated by a wide variety of transporters at the cell membrane level (6), and leads to the alteration of pharmacokinetic profiles. For example, Rugstad *et al.* have reported that pharmacokinetic clearance of piroxicam in men is higher than in women (34). We have reported that rOAT3-mediated uptake of estrone sulfate is strongly inhibited by piroxicam, suggesting that piroxicam may be a substrate of rOAT3 (17). As shown in Fig. 1, rOAT3 mRNA was strongly expressed in the male rat liver and kidney and the female kidney, whereas no rOAT3 mRNA was detected in the female rat liver. Therefore, the sex-dependent pharmacokinetic differences of certain drugs such as piroxicam may be closely related to the expression levels of the OAT3 isoform in the liver. However, further study will be needed to determine whether OAT3 mediates the transport of piroxicam.

In conclusion, we have shown that the OAT isoforms, rOAT2 and rOAT3, are expressed in a sex-related manner whereas there is no sex-dependent expression of the rOAT1 isoform. Additionally, we demonstrated that sex steroids play an important role in the expression of rOAT2/3 isoforms in the liver and kidney. In this respect, further detailed studies will be required to determine whether the involvement of sex steroids in the expression of OAT2 and OAT3 isoforms is only specific to the OAT isoforms or whether this phenomenon is important in the regulation of other transporters in the liver and kidney, such as OAT-K1 (35), OCTN2 (36, 37), oatp (38–40), LST-1 (41, 42), Ntcp (43) and NaDC-1 (44, 45). The results of our study would provide a more complete understanding of the physiological roles of organic anion transporters in the liver and kidney and could explain unexpected side effects in males and females treated with anionic drugs. Further, our results should provide for the development of drugs with desirable pharmacokinetic profiles between men and women by taking into consideration sex-specific differences in expression and regulation of drug transporters. However, such differential gene expressions of OAT isoforms at the protein level and molecular regulation of sex-dependent expressions of these isoforms remain to be investigated.

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