Sex Hormone Regulation of Rat Organic Anion Transporter 3 (rOAT3) Expression in Rat Kidney

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The effects of sex hormones on the level of rat organic anion transporter 3 (rOAT3) protein and its localization were studied in rat kidney. Western blot analysis detected both minor 100-kDa and major 69-kDa proteins in the crude plasma membrane fraction of the kidney. The sum of the levels of both proteins in male rats was not different from that in castrated rats, testosterone-treated rats that had been castrated or female rats. In ovariectomized female rats, the level of these proteins was 3–4 times higher than that in other groups. Treatment of ovariectomized female rats with estradiol reduced rOAT3 to a normal level. Immunohistochemical analysis indicated that rOAT3 was expressed in the renal cortex and outer medulla, where this protein localized in the basolateral membrane of tubules. The immunological localization of rOAT3 was similar in the 6 experimental groups. These results suggest that the cellular level of rOAT3 protein is regulated, at least in part, by estradiol in rat kidney.

Key words —— rat organic anion transporter 3, sex hormone, immunolocalization, kidney

INTRODUCTION

The renal tubular secretion system is responsible for the elimination of hydrophilic compounds including drugs and their metabolites and other xenobiotic agents, and thus plays a pivotal role in the detoxification of these compounds. This transepithelial transport system involves two steps: one is basolateral uptake from blood and the other is apical removal into the tubular lumen, both of which are carrier-mediated processes.[1] In recent studies, various transporter molecules that mediate the membrane transport of organic anions have been cloned from humans and experimental animals.[2–8] rat organic anion transporter 1 (rOAT1) is predominantly expressed in rat kidney and localized in the basolateral membrane of the proximal tubular cells,[2,3,9] mediating the transport of *p*-aminohippuric acid, cyclic nucleotides, prostanoids, dicarboxylates and other anionic drugs.[2,3] rOAT2 and rOAT3 have been cloned from a rat library as the homologues of rOAT1[4–6] that were localized on the apical surface of tubules and basolateral digitations of the cell membrane of tubules, respectively.[10,11] By contrast, rat organic anion transporting polypeptide 1 is located at the apical membrane and is thought to be responsible for the reabsorption of a number of drug metabolites.[7]

Recently, we have observed that the renal clearance of perfluorooctanoic acid (PFOA), a potent surfactant that used in a variety of industrial processes, was more than 20 times higher in female rats than that in male rats.[12,13] Suppression of the renal clearance of PFOA by probenecid suggested that organic anion transporters are responsible for the urinary excretion of PFOA.[13] A sex-related difference in mRNA expression has been reported in organic anion transporting polypeptide,[14,15] rOAT2,[13,16–18] and OAT-K[13] in rat kidney. Comparison of the renal clearance of PFOA with mRNA levels of various organic anion transporters in the kidney of male and female rats with various sex hormone states suggested that rOAT2 and rOAT3 were likely candidates for renal PFOA transporters.[13] However, there are many examples, showing that mRNA levels do not reflect the corresponding protein levels. In the present study, we compared the expression of rOAT3 protein in the kidney of rats under various hormonal states to answer this question. In addition, we determined the immunological localization of rOAT3 in rat kidney.

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MATERIALS AND METHODS

Materials —— Rabbit anti-rOAT3 antibody was purchased from Transgenic (Kumamoto, Japan); peroxidase-conjugated donkey anti-rabbit IgG was from Amersham Biosciences, Piscataway, NJ, U.S.A.); Cy 3-conjugated goat anti-rabbit IgG was from ZYMED Laboratories, CA, U.S.A.). Normal goat serum was purchased from Dako (Glostrup, Demmark). All chemicals and reagents were of analytical grade.

Animals —— Male and female Wistar rats were purchased from SLC (Hamamatsu, Japan) and acclimatized in a humidity- and temperature (23 ± 2°C)-controlled environment with a 12 hr-light/dark cycle for at least 1 week before use. Some male and female rats were castrated and ovariectomized, respectively, under sodium pentobarbital anesthesia (36 mg/kg body weight) at 6 weeks of age. Two weeks after surgery, castrated male rats and ovariectomized female rats were given a daily subcutaneous injection of testosterone propionate dissolved in corn oil (10 mg/kg body weight) for 5 days and estradiol benzoate (0.2 mg/kg body weight) for 7 days, respectively.

Preparation of Crude Plasma Membrane Fraction —— Rat kidneys were perfused with 0.01 M phosphate buffered saline (PBS) and then dissected. Kidneys were weighed, homogenized with 7 volumes 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.08 μg/ml *p*-toluenesultonyl phenylalanine chloromethylketone and 5 mg/ml aprotinin in a glass-Teflon homogenizer. The homogenates were then centrifuged at $500 \times g$ for 5 min. The supernatant was centrifuged at $6000 \times g$ for 5 min and then removed by pipette. The uppermost layer of the pellet was collected, resuspended in the supernatant and recentrifuged at $6000 \times$ for 5 min. The supernatant was removed and the resultant uppermost layer of the pellet was resuspended and centrifuged at $16000 \times g$ for 5 min. The uppermostlayer of the pellet was resuspended and centrifuged at $16000 \times g$ for 20 min. The resultant fluffy white uppermost layer of the pellet was collected, resuspended in a small volume of homogenizing buffer and stored at –80°C until required. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, IL, U.S.A.) with bovine serum albumin as a standard.

Western Blotting —— Known amounts of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 3% stacking gel. Proteins were electroblotted onto a polyvinylidene difluoride membrane (PVDF, Bio Rad Laboratories, Melville, NY, U.S.A.) using a blotting apparatus (Mini Transblot, Bio-Rad Laboratories) at 40 mA for 16 hr at 4°C. The membranes were blocked for 2 hr in Tris-buffered saline [(TBS), 20 mM Tris–HCl (pH 7.0) and 150 mM NaCl] containing 5%(w/v) skimmed milk followed by washing with TBS. The membranes were then incubated with the rabbit anti-rOAT3 polyclonal antibody (1 : 333 dilution in blocking solution) overnight at 4°C. After washing three times with TBS containing 0.1% Tween 20 (TBS-T), the membranes were incubated with horse-radish peroxidase-conjugated donkey anti-rabbit IgG (1 : 1000 dilution in TBS-T) for 1 hr at room temperature followed by washing three times with TBS-T. Immunoreactivity was detected by the chemiluminescence method (Amersham Biosciences) using an imaging analyzer (LAS-1000, Fuji Film, Tokyo, Japan) and quantification was performed using a computer program (Image Gauge, Fuji Film). A standard curve for rOAT3 was obtained using different amounts of protein (1.7–30 μg protein) from the crude plasma membrane fraction prepared from female rat kidney.

Immunohistochemistry —— Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.), and intravenously injected with heparin (10 U/kg body weight). These animals were perfused with 0.1 M phosphate buffer (PB, pH 7.4) containing 4%(w/v) paraformaldehyde between the aorta and renal artery using a peristaltic pump operated at a rate of 20 ml/min for 20 min. After perfusion, the kidneys were dissected and postfixed with the same solution for 4 hr, and then immersed in PB containing 25%(w/v) sucrose for 12 hr at 4°C. After cryoprotection, frozen sections 30-μm in thickness were prepared using a freezing microtome, and mounted on silane-coated slide glasses and air-dried. The sections obtained from the male and female rats in different hormonal states were incubated on the same plates and viewed under the same conditions to compare the expression levels. Sections were washed with 0.01 M PBS containing 0.1% Triton X-100 for 30 min, and then incubated with blocking solution [0.01 M PBS containing 10% goat serum and 4% Block Ace (Dainippon Seiyaku, Tokyo, Japan)] for 1 hr at room temperature, followed by

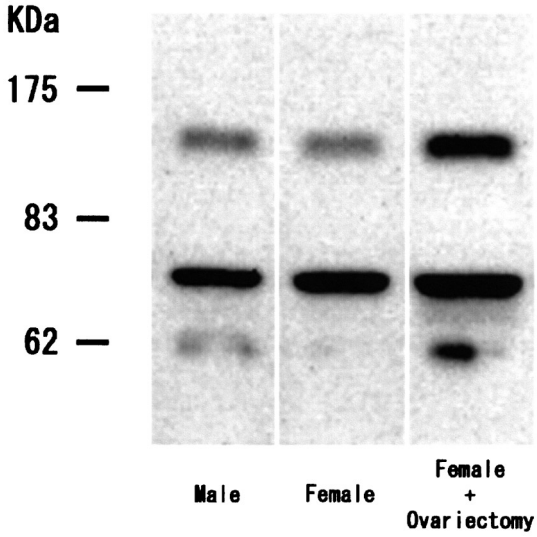

Fig. 1. Western Blot Analysis of rOAT3

Crude plasma membrane fraction prepared from rat kidney (20 μg) was separated by SDS-PAGE (10%) and blotted onto a PVDF membrane. The membrane was incubated with rabbit anti-rOAT3 antibody (0.3 μg/ml) followed by incubation with horseradish peroxidase-conjugated anti rabbit IgG.

overnight incubation with the rabbit anti-rOAT3 polyclonal antibody (1 : 333 dilution in blocking solution) at 4°C. After washing with 0.01 M PBS containing 0.5% Triton X-100, sections were incubated with Cy 3-conjugated goat anti-rabbit IgG (1 : 150 dilution) for 1.5 hr. Microscopic images were obtained using a camera lucida (DP50, Olympus, Tokyo, Japan) and an attached fluorescence microscope (BX51WI, Olympus). Images of the sections were also obtained using a laser-scanning microscope (Zeiss Axioplan, Carl Zeiss, Oberkochen, Germany) equipped with a confocal imaging system (MRC-600 Lasersharp System, Bio Rad Laboratories).

RESULTS AND DISCUSSION

Western blot analysis of the renal membrane fraction for rOAT3 showed a major 69-kDa band and a minor 100-kDa band in the membrane fraction of rat kidney (Fig. 1). The result was not in accordance with the results of Kojima, *et al.*[10] where only a 130-kDa band was detected despite the fact that the same antibody was used in both cases. The reason for the discrepancy remains to be discovered. In the present study, the proportion of 100-kDa and 69-kDa proteins differed from one preparation to another, therefore, we quantified both proteins as rOAT3. In other studies, rOAT3 was detected as a single 65-kDa[11] or 72-kDa protein.[19] It is plausible that the protein is postranslationally glycosylated as has been observed for another transporter.[20] The levels of both 69-kDa and 100-kDa-proteins were significantly higher in ovariectomized female rats than in male rats or female rats (Fig. 1). Next, a standard curve was obtained for the quantification of rOAT3 using different amounts of membrane protein (Fig. 2). A good correlation was observed between the signals for the sum of 69-kDa and 100-kDa proteins and the amounts of protein (Fig. 2C), although a standard curve for 69-kDa or 100-kDa protein alone appeared to be somewhat discontinuous (Figs. 2A and 2B). Next, the relative abundance of rOAT3 protein in the renal membrane fraction was compared between rats in various hormonal states (Fig. 3). No sex-related difference was observed in the level of rOAT3. In addition, no significant difference was observed between control male rats, castrated male rats and testosterone-treated male rats that had been castrated (Fig. 3). By contrast, ovariectomy increased rOAT3 3- to 4-fold and the treatment of ovariectomized rats with estradiol reduced it to the control level (Fig. 3). The levels of rOAT3 protein agreed with the renal levels of rOAT3 mRNA in our previous study.[13] These results suggest that the expression of rOAT3 protein is trasncriptionally controlled.

Immunohistochemical analysis revealed that rOAT3 protein was expressed in the cortex tubules in rat kidney (Fig. 4). As shown in Fig. 4, the level of rOAT3 in the renal cortex was significantly higher than that in other experimental groups. rOAT3 was

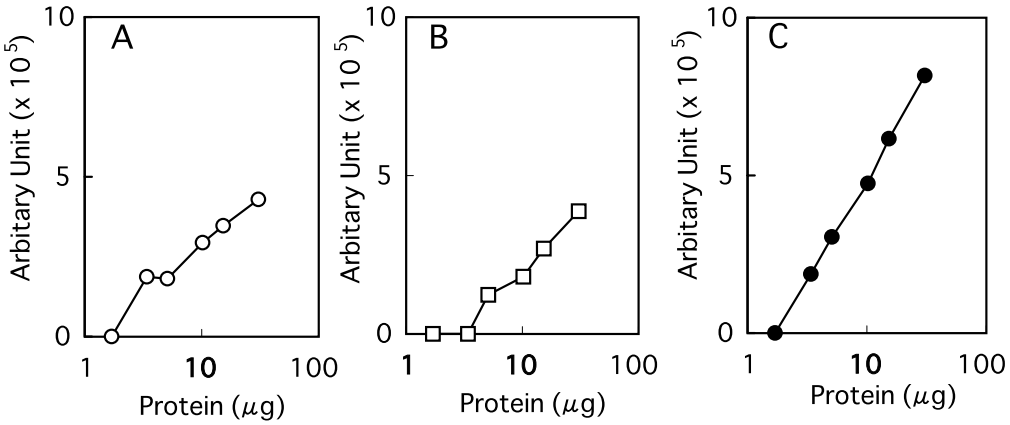

Fig. 2. Standard Curve for Quantification of rOAT3

Different amounts (1.7–30 μg protein) of crude plasma membrane fraction prepared from rat kidney were separated by SDS-PAGE followed by primary and secondary antibodies as described in the legend to Fig. 1. Chemiluminescence was detected by a luminoimage analyzer. A, 100 kDa band; B, 69 kDa band; C, sum of 100 kDa and 69 kDa bands.

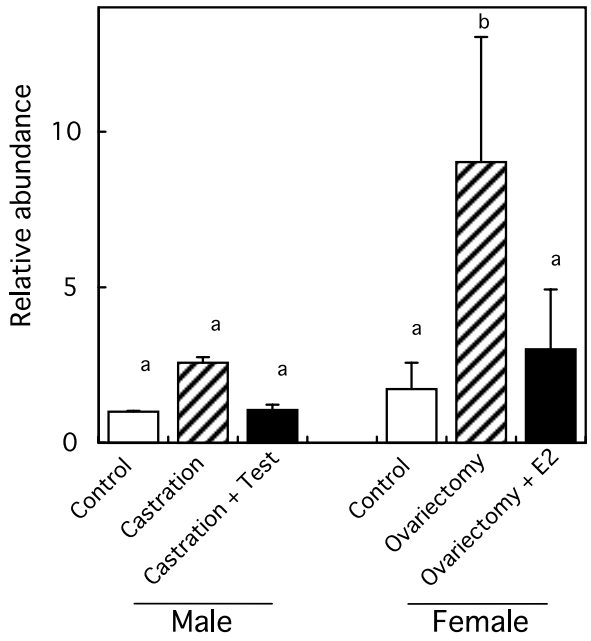

Fig. 3. Renal Expression of rOAT3 Protein in Various Hormonal States

rOAT3 levels were determined in the crude plasma membrane fractions of the kidneys prepared from male rats, castrated male rats, testosterone-treated male rats that had been castrated, female rats, ovariectomized female rats and estradiol-treated female rats that had been ovariectomized. The results are expressed as relative abundance to the value for male rats. Values are means ± S.D. for three rats. Differences between six experimental groups are statistically significant without a common superscript ($p < 0.05$).

also detected in the tubules of the outer medulla where the rOAT3 level was higher in ovariectomized female rats than in the other experimental groups (data not shown). Localization of rOAT3 in the renal cortex and outer medulla has also been reported by Kojima, *et al.*[10] Confocal microscopy at high magnification (× 400) showed that rOAT3 was mainly expressed in the basolateral membrane of tubules in the renal cortex of ovariectomized female rats (Fig. 5).

We have shown that the renal clearance of PFOA is regulated by sex hormones.[12,13] Estimation of the mRNA levels for the transporters responsible for organic anions has suggested that rOAT2 and rOAT3 are candidates for renal PFOA transporters in rats.[13] The mRNA level of rOAT3 was increased by ovariectomy of female rats and this elevated level was reduced to a control level by estradiol treatment.[13]

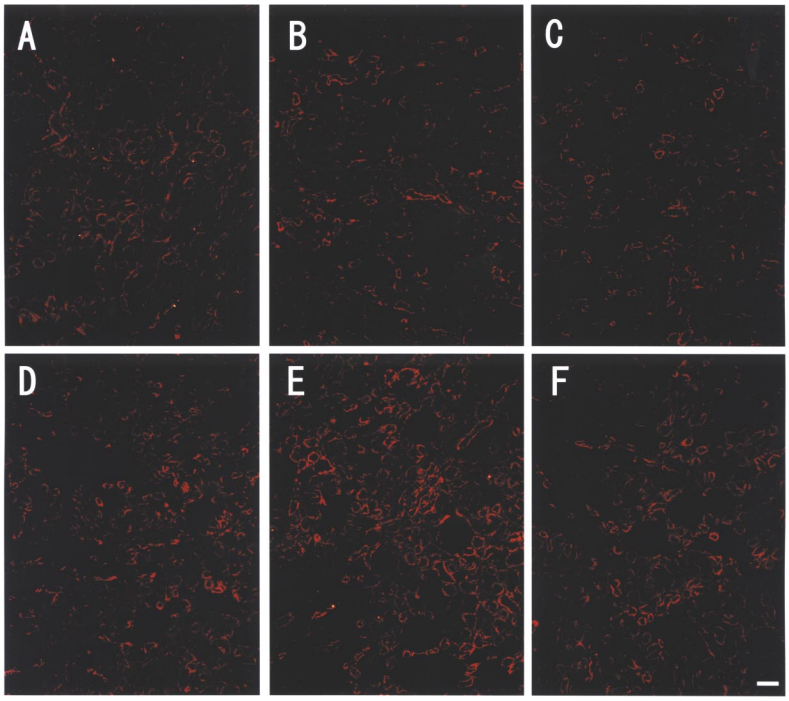

Fig. 4. Immunofluorescence Localization of rOAT3 in the Cortex of Rat Kidney

Cryosections of kidney from a male rat (A), a castrated male rat (B), a testosterone-treated male rat that had been castrated (C), a female rat (D), an ovariectomized female rat (E) and an estradiol-treated female rat that had been ovariectomized (F) were incubated with rOAT3 antibody and stained with Cy 3-conjugated anti rabbit IgG. Scale bar, 40 μm; Magnification, × 100.

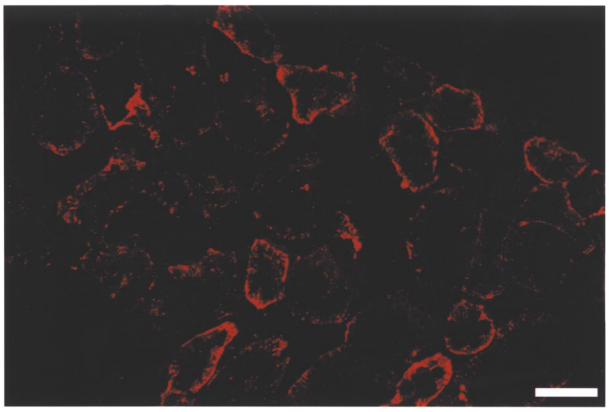

Fig. 5. Immunofluorescence Localization of rOAT3 in the Cortex of Rat Kidney Viewed at High Magnification (× 400)

The renal cortex of an ovariectomized female rat was examined by confocal microscopy. Basolateral localization of rOAT3 was observed in renal tubules. Scale bar, 20 μm; Magnification, × 400.

The present study indicates that rOAT3 protein is actually increased in the basolateral membrane of renal tubules following ovariectomy of female rats. These results suggest that rOAT3 is involved in the uptake of PFOA from the blood and facilitates its urinary excretion.

REFERENCES

1) Inui, K., Masuda, S. and Saito, H. (2000) Cellular and molecular aspects of drug: transport in the kidney. *Kidney Int.*, **58**, 994–958.
2) Sekine, T., Watanabe, N., Hosoyamada, M., Kanai, Y. and Endou, H. (1997) Expression cloning and characterization of a novel multispecific organic anion transporter. *J. Biol. Chem.*, **272**, 18526–18529.
3) Sweet, D. H., Wolff, N. A. and Prichard, J. B. (1997) Expression cloning and characterization of ROAT1. The basolateral organic anion transporter in rat kidney. *J. Biol. Chem.*, **272**, 30088–30095.
4) Simonson, G. D., Vincent, A. C., Roberg, K. J., Huang, Y. and Iwanji, V. (1994) Molecular cloning and characterization of a novel liver-specific transport protein. *J. Cell Sci.*, **107**, 1065–1072.
5) Sekine, T., Cha, S. H., Tsuda, M., Apiwattanakul, N., Nakajima, N., Kanai, Y. and Endou, H. (1998) Identification of multispecific organic anion transporter 2 expressed predominantly in the liver. *FEBS Lett.*, **429**, 179–182.
6) Kusuhara, H., Sekine, T., Utsunomiya-Tate, N., Tsuda, M., Kojima, R., Kanai, Y. and Endou, H. (1999) Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. *J. Biol. Chem.*, **274**, 13675–13680.

7) Jaaquemin, E., Hagenbuch, B., Stieger, B., Wolkoff, A. W. and Meier, P. J. (1994) Expression cloning of a rat liver Na+-independent organic anion transporter. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 133–137.
8) Saito, H., Masuda, S. and Inui, K. (1996) Cloning and functional characterization of a new multispecific organic anion transporter mediating basolateral uptake of methotrexate in the kidney. *J. Biol. Chem.*, **27**, 20719–20725.
9) Tojo, A., Sekine, T., Nakajima, N., Hosoyamada, M., Kanai, Y., Kimura, K. and Endou, H. (1999) Immunohistochemical localization of multispecific renal organic anion transporter 1 in rat kidney. *J. Am. Soc. Nephrol.*, **10**, 464–471.
10) Kojima, R., Sekine, T., Kawachi, M., Cha, S. H., Suzuki, Y. and Endou, H. (2002) Immunolocalization of multispecific organic anion transporters, OAT1, OAT2, and OAT3, in rat kidney. *Am. J. Nephrol.*, **13**, 848–857.
11) Hasegawa, M., Kusuhara, H., Sugiyama, D., Ito, K., Ueda, S., Endou, H. and Sugiyama, Y. (2002) Functional involvement of rat organic anion transporter 3 (rOAT3; slc22a8) in the renal uptake of organic anions. *J. Pharmacol. Exp. Ther.*, **300**, 746–753.
12) Kudo, N., Suzuki, E., Katakura, M., Ohmori, K., Noshiro, R. and Kawashima, Y. (2001) Comparison of the elimination between perfluorinated fatty acids with different carbon chain length in rats. *Chem. -Biol. Interact.*, **134**, 203–216.
13) Kudo, N., Katakura, M., Sato, Y. and Kawashima, Y. (2002) Sex hormone-regulated renal transport of perfluorooctanoic acid. *Chem.-Biol. Interact.*, **139**, 301–316.
14) Lu, R., Kanai, N., Bao, Yi, Wolkoff, A. W. and Schuster, V. L. (1996) Regulation of renal oatp mRNA expression by testosterone. *Am. J. Physiol.*, **270**, F332–F337.
15) Isern, J., Hagenbuch, B., Stieger, B., Meier, P. and Meseguer, A. (2001) Functional analysis and androgen-regulated expression of mouse organic anion transporting polypeptude 1(oatp1) in the kidney. *Biochim. Biophys. Acta*, **1518**, 73–78.
16) Buist, S. C., Cherrington, N. J., Choudhuri, S., Hartley, D. P. and Klaasen, C. D. (2002) Gender-specific and developmental influence on the expression on rat organic anion transporters. *J. Pharmacol. Exp. Ther.*, **301**, 145–151.
17) Kobayashi, Y., Hirokawa, N., Ohshiro, N., Sekine, T., Sasaki, T., Tokuyama, S., Endou, H. and Yamamoto, T. (2002) Differential gene expression of organic anion transporters in male and female rats. *Biochem. Biophys. Res. Commun.*, **290**, 482–487.
18) Kato, Y., Kuge, K., Kusuhara, H., Meier, J. P. and Sugiyama, Y. (2002) Gender difference in the urinary excretion of organic anions in rats. *J. Pharmacol. Exp. Ther.*, **302**, 483–489.
19) Ji, L., Masuda, S., Saito, H. and Inui, K. (2002) Down-regulation of rat organic cation transporter rOCT2 by 5/6 nephrectomy. *Kidney Int.*, **62**, 514–524.
20) Konig, J., Cui, Y., Nies, A. T. and Keppler, D. (2000) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am. J. Physiol.*, **257**, G156–G164.