Gender differences in expression of organic cation transporter OCT2 in rat kidney

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Abstract The organic cation transporter (OCT) mediates translocation of various cationic molecules including drugs, toxins and endogenous substances. We examined gender differences in the expression of rat (r) OCT2 in the kidney. Slices and basolateral membrane vesicles of male rat kidney showed a higher transport activity for tetraethylammonium than those of female rat kidney. The expression levels of rOCT2 mRNA and protein in the kidney of males were much higher than those in females. There was no gender difference in mRNA expression of rOCT1 and rOCT3. These findings suggest that rOCT2 is responsible for the gender differences in renal basolateral membrane organic cation transport activity.

Key words: Organic cation transporter; Tetraethylammonium; Basolateral membrane; Renal tubular secretion; (Rat kidney)

1. Introduction

Renal organic cation transport systems regulate the homeostasis of various positively charged organic solutes including xenobiotics and endogenous substances by mediating tubular secretion and reabsorption [1–3]. Functional studies using isolated membrane vesicles [4–7] and cultured renal epithelial cells [8] suggested that renal tubular secretion of cationic substances is operated effectively by a concerted function of two distinct classes of organic cation transporters: one facilitated by the transmembrane potential difference in the basolateral membranes and the other driven by the transmembrane H^+ gradient in the brush-border membranes. After the first organic cation transporter, rOCT1, was cloned from rat kidney [9], several other members of a common OCT gene family have been identified including OCT2 [10], OCT2p [11], and OCT3 [12]. rOCT1A has been identified from rat intestine as a splice variant of rOCT1 [13]. We identified the second member of the OCT family, rOCT2, in rat kidney, which shows 67% amino acid identity with rOCT1 and transports various cationic solutes such as tetraethylammonium (TEA), cimetidine and guanidine [10,14,15]. There are marked differences in tissue distribution of OCT1, OCT2 and OCT3. rOCT1 was expressed abundantly in the liver and kidney, whereas rOCT2 was found predominantly in the kidney [10], and in dopamine-rich brain areas of rats [11]. rOCT3 has 48% amino acid identity with rOCT1 and is expressed most abundantly in the placenta, but is also detected in the intestine, heart, brain, lung and very weakly in kidney [12]. Functional studies using *Xenopus* oocytes [9,10,15,16] and transfectants of mammalian kidney cells [14,17,18] suggested that both OCT1 and OCT2 from different species are multispecific facilitative transporters which mediate translocation of small organic cations, such as TEA, 1-methyl-4-phenylpyridinium (MPP), N^1-methylnicotinamide (NMN), choline and dopamine, and that both transporters depend on a transmembrane potential difference. Therefore, rOCT1 and rOCT2 have transport properties peculiar to the basolateral membrane transporters of renal proximal tubular cells, although their distributions along the nephron segments are suggested to be different [3]. To gain further insight into the physiological roles of the OCT gene family in renal secretion of xenobiotics, comparative studies are required to show correlations between the organic cation transport activity and expression levels of the cloned transporters in the kidney.

It was demonstrated that renal transport of *p*-aminohippurate (PAH) is higher in male rats than in female rats [19]. Reyes et al. [20] suggested that testosterone could be responsible for the gender differences in renal secretion of PAH by affecting the number of functional transporter proteins. Bowman and Hook [21] reported that the uptake of PAH and TEA by renal cortical slices of male rats was greater than that by female rats, suggesting gender differences in the basolateral membrane transport capacities for both organic anions and cations. There is no information available on the molecular mechanisms underlying the gender differences observed in renal transport of organic ions.

This study was undertaken to examine whether rOCT1 and/or rOCT2 contribute to the gender differences in organic cation transport activity in rat kidney. We report here that rOCT2 should be responsible for the gender differences in organic cation transport of the renal basolateral membranes.

2. Materials and methods

2.1. Materials

[1-^{14}C]TEA bromide (185.0 MBq/mmol) and *p*-[glycyl-^{14}C]aminohippurate (PAH) (1.6 GBq/mmol) were obtained from Du Pont-New England Nuclear Research Products (Boston, MA, USA). HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) was purchased from Nacalai Tesque (Kyoto, Japan). Testosterone and estradiol were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest purity available.

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Abbreviations: OCT, organic cation transporter; OAT, organic anion transporter; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Ntcp, sodium taurocholate transporting polypeptide; oatp, organic anion transporting polypeptide

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2.2. Uptake study by kidney slices

Slices of whole kidneys from male and female Wistar rats (8 weeks old, 190–230 g body weight) were put in ice-cold oxygenated incubation buffer consisting of 120 mM NaCl, 16.2 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.5 as described previously [22]. Slices, each weighing 40–90 mg, from three rats were randomly selected and placed for incubation in a flask containing 3 ml of the incubation buffer with [^{14}C]TEA (50 μM, 0.89 kBq/ml) or [^{14}C]PAH (5 μM, 0.74 kBq/ml). The uptake of [^{14}C]TEA or [^{14}C]PAH was carried out at 25°C under an atmosphere of 100% oxygen. D-[^{3}H]Mannitol (50 μM or 5 μM, 3.7 kBq/ml) was used to estimate the extracellular trapping and non-specific uptake of [^{14}C]TEA or [^{14}C]PAH. After incubation for an appropriate time, each slice was rapidly removed from the flask, washed twice in 3 ml of ice-cold incubation medium, blotted on filter paper, weighed, and solubilized in 0.5 ml of NCS II (Amersham International, Buckinghamshire, UK). The radioactivity was determined in 5 ml of ACS II (Amersham) by liquid scintillation counting.

2.3. Uptake study by basolateral membrane vesicles

Basolateral membrane vesicles were isolated from the kidneys of male and female Wistar rats (8 weeks old, 190–230 g body weight) according to the method of Percoll density gradient centrifugation [4]. The purified basolateral membranes were suspended in buffer comprising 100 mM D-mannitol, 10 mM HEPES (pH 7.5), and 100 mM KCl (buffer A). The uptake of [^{14}C]TEA by membrane vesicles was measured by a rapid filtration technique [4]. Briefly, the uptake was rapidly initiated by addition of buffer A containing [^{14}C]TEA (20 μl) to 20 μl of membrane suspension at 37°C. At the stated times, incubation was terminated by diluting the reaction mixture with 1 ml of ice-cold stop solution. The stop solution contained 150 mM KCl and 20 mM HEPES-Tris (pH 7.5). The contents of the tube were poured immediately onto Millipore filters (HAWP, 0.45 μm), washed once with 5 ml of ice-cold stop solution and counted in 5 ml of ACS II by liquid scintillation counting. The data are presented as specific uptake activity which was inhibited in the presence of 10 mM unlabeled TEA. The protein amount in vesicles was determined by the method of Bradford [23], using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine γ-globulin as standard.

2.4. Northern blot hybridization

Total RNA was extracted from whole kidney using the guanidine/isothiocyanate method [24]. In brief, 10 μg of total RNA was electrophoresed in 1% denaturing agarose gel containing formaldehyde and transferred onto nylon membranes. The quality of RNA was assessed by ethidium bromide staining. After transfer, blots were hybridized at high stringency (50% formamide, 5×SSPE [1×SSPE = 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA], 5×Denhardt's solution, 0.2% SDS, 10 μg/ml salmon sperm DNA at 42°C) with cDNA encoding either rOCT1, rOCT2, rOAT1 or rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) labeled with [α-^{32}P]dCTP. The cDNA probes corresponded to the nucleotide positions at 399–1882 (rOCT1), 362–2114 (rOCT2), 966–1773 (rOCT3), 1–2227, (rOAT1) and 10–1047 (GAPDH) of the published sequences in the GenBank/EBI Data Bank. After hybridization, the blots were washed twice in 2×SSC (1×SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0)/0.5% SDS at room temperature for 10 min, and then twice in 0.2×SSC/0.5% SDS at 65°C for 30 min. Dried membranes were exposed to the imaging plates of FUJIX BIO-Imaging Analyzer BAS 2000 II (Fuji Photo Film).

2.5. Polyclonal antibodies against rat OCT2 and Western blot analysis

According to the previously reported methods [25], polyclonal antibodies were raised against a synthetic peptide corresponding to the intracellular domains of rOCT2 (LTPDEDAGKKLKPSI) in New Zealand White rabbits. The peptide was synthesized with cysteine at its NH_2-terminal and conjugated to keyhole limpet hemocyanin. Crude plasma membrane fractions were prepared from rat kidneys as reported previously [26]. The membrane fractions were solubilized in lysis buffer (2% SDS, 125 mM Tris, 20% glycerol). The samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) by semi-dry electroblotting. The blots were blocked with 5% non-fat dry milk and 5% bovine serum albumin in phosphate-buffered saline containing 0.5% Tween 20 (PBS-T; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.5), and left overnight at 4°C. The blots were washed three times in PBS-T and incubated with the anti-rOCT2 antiserum (1:1000) for 2 h at 25°C. The blots were washed three times with PBS-T, and the bound antibody was detected on X-ray film by enhanced chemiluminescence (ECL) with a horseradish peroxidase-conjugated anti-rabbit IgG antibody and cyclic diacylhydrazides (Amersham).

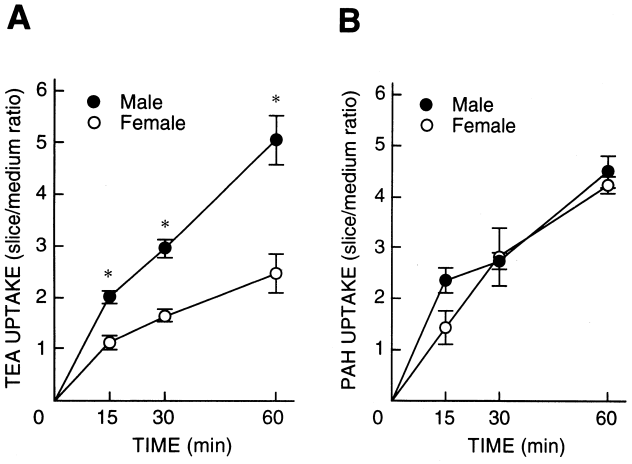

Fig. 1. Accumulation of TEA and PAH by slices of male and female rat kidneys. Renal slices were incubated at 25°C in buffer containing 50 μM [^{14}C]TEA (A) or 5 μM [^{14}C]PAH (B) for the indicated periods. D-[^{3}H]Mannitol was used to estimate the extracellular trapping and non-specific uptake of [^{14}C]TEA and [^{14}C]PAH. Each point represents the mean ± S.E.M. of three slices from a typical experiment. *$P < 0.05$, significantly higher than female.

2.6. Statistical analysis

Statistical analysis was performed by the Student's *t*-test, or by the one-way analysis of variance followed by Fisher's *t*-test, when multiple comparisons against the control were needed.

3. Results

First, in order to gain information about the gender differences in organic cation transport activity, we examined accumulation of TEA by slices of the kidneys from male and female rats. Fig. 1 shows the time course for accumulation of TEA and PAH. The TEA accumulation rate was approx-

Table 1
Uptake of [^{14}C]TEA by basolateral membrane vesicles isolated from male and female rat kidneys

Male (pmol/mg of protein/30 s)		Female (pmol/mg of protein/30 s)	
Cortex	Medulla	Cortex	Medulla
336.2 ± 37.0	210.7 ± 22.6*	232.2 ± 45.3	124.7 ± 5.2

Basolateral membrane vesicles from cortex and medulla of rat kidneys were incubated with [^{14}C]TEA for 30 s at 37°C. Each point represents the mean ± S.E.M. of three separate experiments performed in three determinations. TEA uptake by the vesicles from medulla of male rat kidney was significantly greater than that of female rat kidney (*$P < .05$).

imately twofold greater in renal slices of male rats than female rats (Fig. 1A), which was comparable to the finding reported previously [21]. In contrast, there was no significant difference in PAH accumulation by male and female renal slices (Fig. 1B).

To ascertain if there were gender differences in TEA transport activity of the renal basolateral membranes, uptake of TEA was examined using isolated basolateral membrane vesicles from male and female kidneys. Consistent with the results for TEA accumulation by renal slices (Fig. 1A), TEA uptake was greater in the vesicles of male kidney compared to that of female kidney (Table 1). TEA uptake by the vesicles from cortex of male kidney showed a tendency to be higher than that of female kidney.

Total RNA from male and female kidneys was analyzed by Northern blot hybridization for the mRNA expressions of rOCT1, rOCT2, rOCT3 and rOAT1. An mRNA of 1.9 kb was detected for rOCT1, and there was no appreciable difference in abundance of the transcript between male and female kidneys (Fig. 2). In contrast, the amount of the rOCT2 mRNA of 2.2 kb was much lower in females than in males. The mRNA level of GAPDH did not differ between males and females. The signal for rOCT3 mRNA of 3.5 kb was very weak and showed no difference in abundance of the transcript between male and female kidneys. The amount of the rOAT1 mRNA, a kidney-specific organic anion transporter [27], also did not differ between male and female kidneys.

Crude plasma membranes isolated from male rat tissues were subjected to immunoblot analysis for rOCT2 protein. A primary band with a size of 74 kDa (calculated molecular weight of 66 kDa) was detected exclusively in the membranes of kidney (Fig. 3A), and was more intense in the medulla than in the cortex. Fig. 3B shows rOCT2 protein in the kidneys of male and female rats. The density of the band for male kidney was much more intense than for female kidney. The rOCT2 protein of 74 kDa disappeared when the antibody was preabsorbed to the synthetic antigen peptide (data not shown).

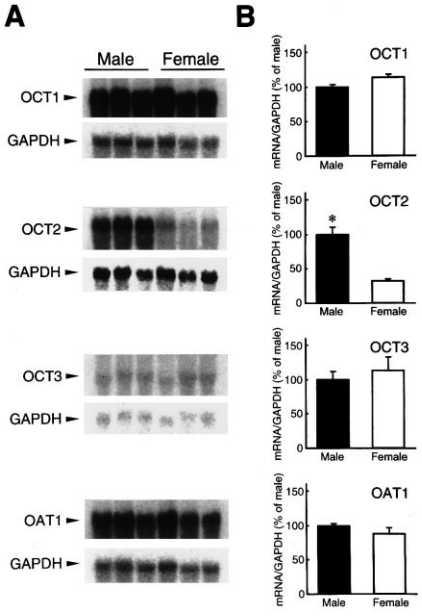

Fig. 2. Northern blot analysis of total RNA from male and female rat kidneys. A: Total RNA (10 μg) from male and female rat kidneys, hybridized with rOCT1, rOCT2, rOCT3, rOAT1 or GAPDH cDNA probe under high stringency. B: Densitometric quantitation of rOCT1, rOCT2, rOCT3 and rOAT1, corrected for loading using GAPDH. Male levels were set at 100%. No differences in GAPDH were measured between male and female rats. Each column represents mean ± S.E.M. of three rats. $*P < 0.05$, significantly higher than female.

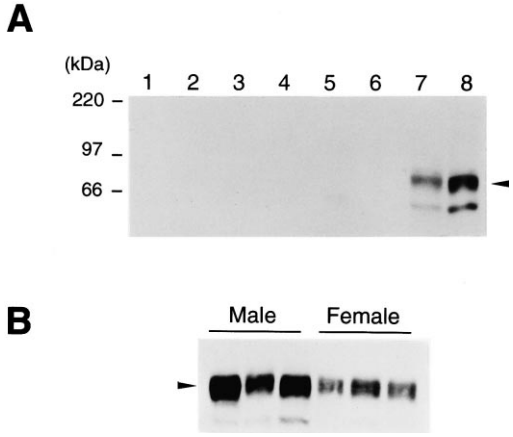

Fig. 3. Immunoblot analysis of membrane proteins from male and female rat kidneys. A: Crude membranes (20 μg) isolated from brain (lane 1), heart (lane 2), lung (lane 3), liver (lane 4), small intestine (lane 5), spleen (lane 6), kidney cortex (lane 7), and kidney medulla (lane 8) were separated on SDS-PAGE. B: Crude membranes (50 μg) from the kidneys of three male and three female rats were separated on SDS-PAGE. rOCT2 was identified using polyclonal antibodies as described in Section 2. The arrows indicate the position of rOCT2.

4. Discussion

This study demonstrates that the gender difference in the expression of rOCT2 in the kidney is responsible for the gender differences in organic cation transport activity of the basolateral membrane of renal tubular cells. Gender differences in organic anion and cation transport activities of rat kidney have been described by Bowman and Hook [21], demonstrating that accumulation of TEA was greater in renal cortical slices from male than female rats, which is consistent with the present findings (Fig. 1). They concluded that male rat kidney had a more effective transport system for TEA than female rat kidney. Since uptake by renal slices is considered to reflect predominantly transport across the basolateral membranes, the gender differences in TEA transport could result from differences in the expression of basolateral membrane transporter(s).

rOCT1, rOCT2 and rOCT3 have been shown to be expressed in the kidney [9,10,12]. Functional studies suggested that these OCT gene members possess transport characteristics peculiar to those of the basolateral membrane transport system for small organic cations [4,7,8]. It is noteworthy that rOCT1 mRNA is abundantly expressed in the liver as well as in the kidney, and that OCT1 mRNA is not detected in the human kidney [28], probably due to species differences. In contrast, rOCT2 mRNA is expressed predominantly in the kidney [10], and in neurons [11]. Initially, we determined the precise amount of mRNA for rOCT1 and rOCT2 in rat kidney, and observed that the steady-state mRNA level of rOCT2 was sixfold higher than that of rOCT1 (unpublished data). To date, there is no information available on the gender

differences and hormonal regulation of OCT gene family expression.

In the present study, Northern blot and immunoblot analyses revealed that the amounts of both rOCT2 transcript and protein expression were greater in the kidneys of male rats than of female rats (Figs. 2 and 3). In contrast, there were no appreciable gender differences in the mRNA expression of rOCT1, rOCT3 and rOAT1 (Fig. 2). Thus, we assumed that rOCT2 could be an organic cation transporter whose expression is regulated by gender-related factors, whereas rOCT1 may be a constitutive type of the transporter.

Gender differences in the hepatic transport of endogenous organic anions including taurocholate [29] and estradiol 17β-glucuronide [30], and the mechanisms involved in these differences have been described. Simon et al. [29] suggested that the protein content of Ntcp, the sodium taurocholate transporting polypeptide, was significantly greater in male liver, and that the expression of Ntcp was transcriptionally controlled. Also, the studies demonstrated that sex steroid hormones possibly working with growth hormones may contribute to the regulation of hepatic sinusoidal transport of taurocholate. Endogenous hormones responsible for the regulation of Ntcp mRNA expression have not yet been identified. Lu et al. [30] reported that the steady-state levels of mRNA expression of renal but not hepatic organic anion transporting polypeptide (oatp) were strongly regulated by testosterone and, to a lesser extent, estradiol. In the present study, we determined the steady-state rOCT2 mRNA levels by Northern blot hybridization. Thus, it has not been clarified whether the gender differences in rOCT2 mRNA expression are transcriptional or posttranscriptional. Further studies such as nuclear run-on analysis and mRNA stabilities, and promoter identification are needed to address this issue. Treatment of rats with testosterone and estradiol may also provide information on the transcriptional regulation of rOCT2 expression. Although it is still unclear why the expression of rOCT2, but not of rOCT1 or rOCT3, is regulated sexually in the kidney, we speculate that this transporter might play a physiological role in the metabolic clearance of endogenous substances, which may be generated differentially in males and females, for effective removal from the circulation to maintain hormone balance.

By functional studies using *Xenopus* oocytes and stable transfectants, we found that rOCT1 and rOCT2 possess similar but not identical affinities for a variety of compounds [14,15]. The physiological implication of the difference in roles of these transporters in the renal handling of organic cations has not been clarified. Considering the present findings, it is likely that rOCT1 and rOCT2 share the metabolic clearance of small cationic solutes in the kidney, and that rOCT2-mediated cation uptake could be modulated by endogenous factor(s). The reason why rOCT1, but not rOCT2, is expressed not only in the kidney but also in the liver might be related to the functional role of each transporter in the homeostasis of cationic substances.

In conclusion, we demonstrated that rOCT2 is responsible for the gender differences in renal organic cation transport activity, possibly in the basolateral membranes of tubular epithelial cells. These findings suggest that rOCT2 may play physiological and pharmacological roles in renal clearance of endogenous and exogenous organic cations from the blood.

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References

[1] Pritchard, J.B. and Miller, D.S. (1993) Physiol. Rev. 73, 765–796.
[2] Inui, K. and Okuda, M. (1998) Clin. Exp. Nephrol. 2, 100–108.
[3] Koepsell, H., Gorboulev, V. and Arndt, P. (1998) J. Membr. Biol. 167, 103–117.
[4] Inui, K., Okano, T., Takano, M., Kitazawa, S. and Hori, R. (1981) Biochim. Biophys. Acta 647, 150–154.
[5] Holohan, P.D. and Ross, C.R. (1981) J. Pharmacol. Exp. Ther. 216, 294–298.
[6] Hsyu, P.-H. and Giacomini, K.M. (1987) J. Biol. Chem. 262, 3964–3968.
[7] Sokol, P.P. and Mckinney, T.D. (1990) Am. J. Physiol. 258, F1599–F1607.
[8] Saito, H., Yamamoto, M., Inui, K. and Hori, R. (1992) Am. J. Physiol. 262, C59–C66.
[9] Gründemann, D., Gorboulev, V., Gambaryan, S., Veyhl, M. and Koepsell, H. (1994) Nature 372, 549–552.
[10] Okuda, M., Saito, H., Urakami, Y., Takano, M. and Inui, K. (1996) Biochem. Biophys. Res. Commun. 224, 500–508.
[11] Gründemann, D., Babin-Ebell, J., Martel, F., Örding, N., Schmidt, A. and Schömig, E. (1997) J. Biol. Chem. 272, 10408–10413.
[12] Kekuda, R., Prasad, P.D., Wu, X., Wang, H., Fei, Y.-J., Leibach, F.H. and Ganapathy, V. (1998) J. Biol. Chem. 273, 15971–15979.
[13] Zhang, L., Dresser, M.J., Chun, J.K., Babbitt, P.C. and Giacomini, K.M. (1997) J. Biol. Chem. 272, 16548–16554.
[14] Urakami, Y., Okuda, M., Masuda, S., Saito, H. and Inui, K. (1998) J. Pharmacol. Exp. Ther. 287, 800–805.
[15] Okuda, M., Urakami, Y., Saito, H. and Inui, K. (1999) Biochim. Biophys. Acta 1417, 224–231.
[16] Busch, A.E., Quester, S., Ulzheimer, J.C., Waldegger, S., Gorboulev, V., Arndt, P., Lang, F. and Koepsell, H. (1996) J. Biol. Chem. 271, 32599–32604.
[17] Busch, A.E., Quester, S., Ulzheimer, J.C., Gorboulev, V., Akhoundov, A., Waldegger, S., Lang, F. and Koepsell, H. (1996) FEBS Lett. 395, 153–156.
[18] Martel, F., Vetter, T., Russ, H., Gruündemann, D., Azevedo, I., Koepsell, H. and Schömig, E. (1996) Naunyn-Schmiedeberg's Arch. Pharmacol. 354, 320–326.
[19] Kleinman, L.I., Lowenstein, M.S. and Goldstein, L. (1966) Endocrinology 78, 403–406.
[20] Reyes, J.L., Meléndez, E., Alegría, A. and Jaramillo-Juarez, F. (1998) Endocrinology 139, 1581–1587.
[21] Bowman, H.M. and Hook, J.B. (1972) Proc. Soc. Exp. Biol. Med. 141, 258–262.
[22] Hori, R., Ishikawa, Y., Takano, M., Okano, T., Kitazawa, S. and Inui, K. (1982) Biochem. Pharmacol. 31, 2267–2272.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[25] Saito, H., Okuda, M., Terada, T., Sasaki, S. and Inui, K. (1995) J. Pharmacol. Exp. Ther. 275, 1631–1637.
[26] Ogihara, H., Saito, H., Shin, B.-C., Terada, T., Takenoshita, S., Nagamachi, Y., Inui, K. and Takata, K. (1996) Biochem. Biophys. Res. Commun. 220, 848–852.
[27] Sekine, T., Watanabe, N., Hosoyamada, M., Kanai, Y. and Endou, H. (1997) J. Biol. Chem. 272, 18526–18529.
[28] Gorboulev, V., Ulzheimer, J.C., Akhoundova, A., Ulzheimer-Teuber, I., Karbach, U., Quester, S., Baumann, C., Lang, F., Busch, A.E. and Koepsell, H. (1997) DNA Cell Biol. 16, 871–881.
[29] Simon, F.R., Fortune, J., Iwahashi, M., Bowman, S., Wolkoff, A. and Sutherland, E. (1999) Am. J. Physiol. 276, G556–G565.
[30] Run, L., Kanai, N., Bao, Y., Wolkoff, A.W. and Schuster, V.L. (1996) Am. J. Physiol. 270, F332–F337.