Discussion

Attenuated diuretic response is frequently observed in cases of chronic administration of furosemide (Green and Mirkin, 1980; Keller et al., 1982; Smith et al., 1985; Kirchner et al., 1990; Kirchner et al., 1992; Wilcox, 2002). It is thought that a possible cause of this attenuation is a decrease of circulating blood volume and glomerular filtration rate, which leads to a decrease in the concentration of sodium ions in the thick ascending limb of Henle's loop and a compensating increase of sodium reabsorption in the distal tubule (Loon et al., 1989). However, in nephrotic syndrome, because a great amount of albumin is present in urine, furosemide may bind to albumin rather than to the Na^+- K^+-$2Cl^-$ cotransporter, decreasing its effectiveness (Green and Mirkin, 1980; Keller et al., 1982; Smith et al., 1985; Kirchner et al., 1990; Kirchner et al., 1992; Wilcox, 2002). This suggests that diuretic resistance can be restored by effective inhibition of furosemide-albumin binding, which should cause an increase of the free concentration of furosemide in renal tubules. The binding inhibitor should possess the following properties: 1) it is a potent inhibitor of the protein binding of furosemide in urine; 2) when administered in large doses, its plasma concentration reaches high levels; 3) it is primarily excreted in urine; and 4) it is highly safe and suitable for prolonged administration. In a previous study, we demonstrated that valproic acid, phenytoin and bucolome, which all bind to site I, inhibited the protein binding of furosemide when administered at typical clinical doses (Takamura et al., 1996). Bucolome, a nonsteroidal anti-inflammatory drug, is usually administered in large doses (600-1200 mg); its plasma concentration is typically about 300 μM; and it is primarily eliminated by urine excretion (Kakemi et al., 1970; Yashiki et al., 1971a; Yashiki et al., 1971b; Chiba et al., 1985). It has been reported that bucolome does not affect urinary enzyme activities, suggesting that any nephrotoxicity of bucolome would be very low-level (Tsurumi et al., 1978). Also, bucolome

has been used to treat nephrotic syndrome patients in warfarin-bucolome combination therapy (Sato et al., 1991). Thus, it appears that bucolome is suitable for treatment of nephrotic syndrome patients, despite the need for careful monitoring of renal function.

The diuretic resistance caused by decreased levels of the active form (i.e., unbound form) of furosemide in urine can be alleviated by inhibiting protein binding of furosemide, which increases the amount of free furosemide delivered to the site of action. In the present study, when bucolome was administered with furosemide in healthy volunteers, the free fraction of furosemide in plasma increased (Fig. 2), as predicted by the *in vitro* data (Fig. 1). The free fractions of some ligands were slightly influenced by the temperature (Melten et al., 1986), suggesting that the present protein binding obtained by ultrafiltration at 25 °C might be more greater than that at 37 °C. However, in terms of the qualitative analysis, it is suggested that bucolome inhibits the binding of furosemide under *in vivo* physiological condition. Therefore, a decrease in the AUC and an increase in the CL_{tot} and V_{dss} would be due to the decrease in the protein binding of furosemide (Table I). Also, the natriuretic effect of furosemide was reinforced by coadministration of bucolome (Table II). We hypothesize that the alterations of these pharmacokinetic parameters were caused by the increase in the amount of furosemide available for the hepatic metabolism and renal excretion, followed by the increase in the free fraction of furosemide. The increase of renal clearance of furosemide resulted in the increase of the amount reaching the site of action (Table I), the Na^+- K^+-$2Cl^-$ cotransporter, and induced the acceleration of the diuretic effect (Table II).

Even though the amount of furosemide reaching the site of action was increased by the coadministration of bucolome, unless the protein binding of furosemide in urine is inhibited, the blunted response to furosemide may not be alleviated in patients with nephrotic syndrome. In the present study, accurate concentrations of drugs tested in the nephrotic loop could not be estimated. However, considering the pharmacokinetic parameters of furosemide

(Table 1) and bucolome (Yashiki et al., 1971a; Yashiki et al., 1971b), their urinary concentrations were expected to reach about 30 and 500 μM, respectively. Additionally, clinical dose of furosemide is often increased in patients with nephrotic syndrome. Therefore, we assumed that urinary concentrations of furosemide and bucolome in the renal tubules were 30 or 60 μM, and 500 μM, respectively (Fig. 3A), although they would be more concentrated than the artificial urine used in this experiment. As a result, we demonstrated that bucolome effectively inhibited the protein binding of furosemide even in urine. Sulfamethizole and sulfisoxazole, which bind to site I of HSA, did not inhibit the protein binding of furosemide in urine, even at doses of 1 mM, although they inhibit binding effectively in plasma (data not shown). This suggests that it is important for the binding inhibitor to possess the high affinity for albumin exhibited by bucolome ($n_I \cdot K_I = 1.5 \times 10^6\ M^{-1}$). In addition, as an inhibitor for protein binding of furosemide in urine, bucolome has the advantage that its effects are not altered by changes in pH (Fig. 3B) or the presence of other drugs including sulfamethizole and sulfisoxazole. The binding percentage of furosemide in an artificial urine was about 70 % (Fig. 3A), which was lower than the percentage in plasma (about 99 %), suggesting that alteration of pH between urine and plasma, and/or endogenous inhibitors, such as fatty acid, uremic toxins and eicosanoids, may be involved in the protein binding of furosemide in the urine (Kragh-Hansen et al., 2002).

Interestingly, we found that coadministration of bucolome with furosemide in adriamycin-induced NS rats alleviated the diuretic resistance (Fig. 4). It has been repeatedly shown that the urinary concentration of loop diuretic is the best index of drug concentration at the intraluminal site of action in the thick ascending limb of Henle's loop. And, there are publications for furosemide that depict urine output versus excretion rates to better reflect the pharmacodynamics (Green and Mirkin, 1980; Keller et al., 1982; Smith et al., 1985; Kirchner et al., 1990; Kirchner et al., 1992; Wilcox, 2002). In the present study, promotion of diuretic

effects by bucolome was accompanied by increasing urinary excretion rate of furosemide (Fig. 4), suggesting an increase in the amount of furosemide delivered to the site of action. These results are in good agreement with the present findings for healthy subjects, suggesting that this therapeutic strategy can restore the diuretic response to furosemide in patients with nephrotic syndrome. The affinity of furosemide for RSA was higher than its affinity for HSA, although bucolome had approximately equal affinity for RSA and HSA. The inhibitory effect of bucolome on the protein binding of furosemide in plasma and urine is likely to be stronger in humans than rats, suggesting that bucolome has a greater effect on the diuretic properties of furosemide in patients with nephrotic syndrome than in NS rats. On the other hand, the mean urine volume attained by the coadministration of bucolome (20 mg/kg) with furosemide in the NS rats (Fig. 4D, 395 to 753 μL) was still less than that observed in normal rats (Fig. 4C, 1352 to 2103 μL), although coadministration of bucolome with furosemide doubled the urinary excretion of furosemide at 3 hours post-dose (Fig. 4, A and B). Thus, the present limited data suggested that coadministration of bucolome with furosemide may partially restore the diuretic resistance in the nephrotic rats. A critical element of the present strategy is to increase the amount of free furosemide delivered to the site of action. In the condition of repeated administration, that is the clinically relevant situation, highly concentrations of furosemide and bucolome would be maintained in the urine. Therefore, the inhibitory effect of bucolome by chronic dosing may be more possibly generated compared with that by single dosing.

The present results suggest that coadministration of bucolome can partially reverse the diuretic resistance of furosemide in patients with nephrotic syndrome. These findings may lead to an effective clinical therapy for alleviation of diuretic resistance of furosemide in nephrotic syndrome.

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Figure legends

Fig. 1. Binding of furosemide in the presence of bucolome (A) and vice versa (B) to HSA at pH 7.4 and 25°C.

(A), Binding of furosemide (10 - 20 μM) to HSA (120 μM) in the presence of bucolome (60 μM). (B), Binding of bucolome (30 - 50 μM) to HSA (120 μM) in the presence of furosemide (60 μM). ●, Experimental values, ------; Theoretical curve assuming competitive binding, ——— , Theoretical curve assuming independent binding. All theoretical curves were constructed using the n_1 and K_1 values (furosemide, $n_1 \cdot K_1 = 2 \times 10^5$ M^{-1} (Takamura et al., 1996); bucolome, $n_1 K_1 = 1.5 \times 10^6 M^{-1}$).

Fig. 2. Serum protein binding of furosemide after single intravenous injection of furosemide to healthy volunteers alone (A) and with bucolome (B).

Serum free fraction of furosemide was determined at 5, 10 and 15 min after single intravenous administration. Each column is the mean of three experiments ± S.D. *: $p < 0.05$ (A vs. B), **: $p < 0.01$ (A vs. B). Statistical analysis was performed by Paired t-test.

Fig. 3. Effect of bucolome on the protein binding of furosemide in UPA.

(A), Concentration-dependent protein binding of furosemide (A and B, 30 μM; C and D, 60 μM) was examined without bucolome (A and C) and with 500 μM bucolome (B and D) at 25°C. Concentration of urinary protein was 480 μM (as HSA). (B), Effect of pH on the protein binding of furosemide was examined in the presence of bucolome in UPA at 25°C. The following concentrations were used: [urinary protein], 480 μM (as HSA); [furosemide], 30 μM; [bucolome], 500 μM. Each bar represents mean ± S.D. (n=3). *: $p <$ 0.001 vs. furosemide alone.

Fig. 4. Urinary excretion of furosemide (A and B) and urine volume (C and D) after i.v. administration of furosemide to normal (A and C) and adriamycin-treated rats (B and D) alone and with bucolome.

Bucolome was orally coadministered (○, 0 mg/kg; ●, 10 mg/kg; ▲, 15 mg/kg; ■, 20 mg/kg) with single intravenous injection of furosemide (2 mg/kg) to normal and adriamycin-treated rats. Each point represents the mean ± S.D. (n = 4-9). *: $p < 0.05$, **: $p < 0.01$ vs. furosemide alone.

Table 1 Kinetic parameters of furosemide after single intravenous injection of furosemide to healthy volunteers alone (A) and with bucolome (B)

	Furosemide alone	With bucolome
AUC (mg/L·h)	2.55 ± 0.22	1.84 ± 0.24***
CL_{tot} (L/h)	7.89 ± 0.60	11.0 ± 1.35*
CL_r (L/h)	3.63 ± 0.57	8.37 ± 1.43**
V_{dss} (L)	5.54 ± 0.96	8.57 ± 1.11**
$t_{1/2\beta}$ (h)	0.67 ± 0.28	0.69 ± 0.26

Each value represents the mean ± S.D. (n = 3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs. furosemide alone.

Table 2 Urine volume and urinary excretion of sodium for 6 hours after single intravenous injection of furosemide to healthy volunteers alone (A) and with bucolome (B)

	Furosemide alone	With bucolome
Urine volume (L)	1.23 ± 0.15	1.48 ± 0.12*
Urinary sodium (mM)	7.89 ± 0.60	11.0 ± 1.35*

Each value represents the mean ± S.D. (n = 3).

*: $p < 0.05$ vs. furosemide alone.

Table 3 Pharmacokinetic parameters of furosemide after single intravenous injection to normal rats (2 mg/kg) alone and with oral coadministration of bucolome

Parameters	Furosemide alone	With bucolome		
		10 mg/kg	15 mg/kg	20 mg/kg
AUC (μg/mL · min)	563 ± 82	572 ± 31	414 ± 47*	336 ± 91**
CL_{tot} (mL/min/kg)	3.61 ± 0.52	3.51 ± 0.19	4.87 ± 0.52	6.24 ± 1.54*
CL_r (mL/min/kg)	1.08 ± 0.16	1.42 ± 0.25	2.11 ± 0.21**	2.67 ± 0.42**
V_{dss} (mL/kg)	75.7 ± 6.0	75.1 ± 1.3	92.1 ± 5.6**	112 ± 7**
$t_{1/2\beta}$ (min)	44.7 ± 12.7	33.6 ± 3.7	35.7 ± 9.6	29.4 ± 4.5
f_b (%)	1.28 ± 0.36	1.41 ± 0.72	1.99 ± 0.76	3.03 ± 0.57**

Each value represents the mean ± S.D. (n = 3-4).

*: $p < 0.05$, *: $p < 0.01$ vs. furosemide alone.

Table 4 Biochemical parameters at 2 weeks after intravenous administration of adriamycin (9 mg/kg)

	Control	With adriamycin
Body weight (g)	203 ± 11	176 ± 16*
Hematocrit (%)	37.4 ± 3.9	35.4 ± 1.7
Serum albumin level (mL/min/kg)	3.75 ± 0.14	3.03 ± 0.23**
Urine volume (ml/day)	6.53 ± 0.47	6.27 ± 3.00
Proteinuria (mg/day)	9.04 ± 4.15	275 ± 84*

Each value represents the mean ± S.D. (n = 3-6).

*: $p < 0.05$, *: $p < 0.01$ vs. control.

Figure 1.

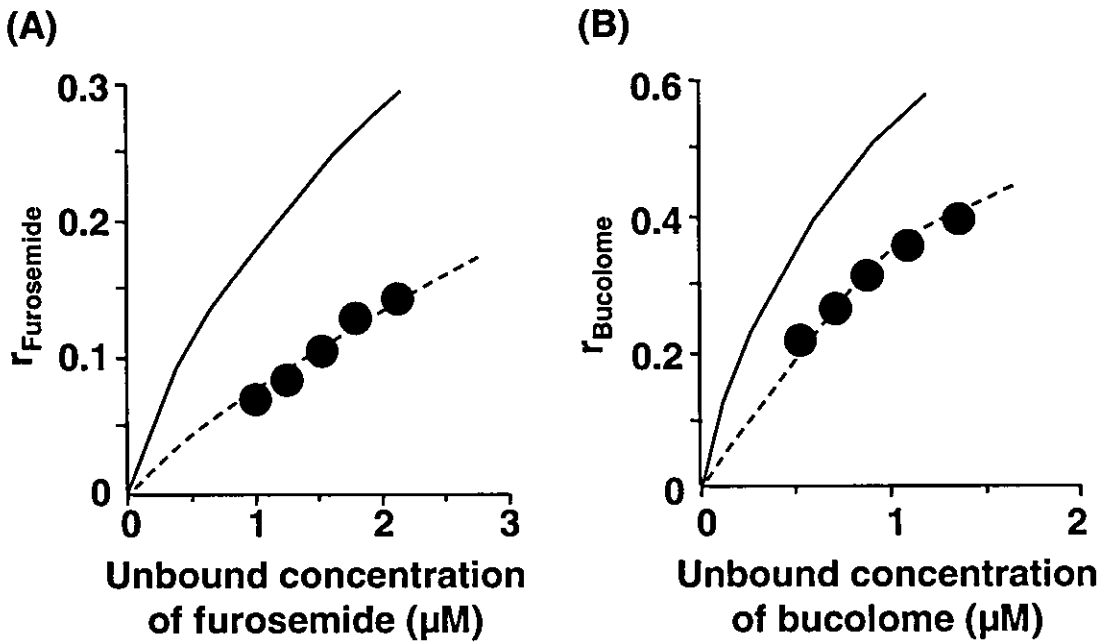

(A)
0.3
0.2
0.1
0
$r_{Furosemide}$
0
1
2
3
Unbound concentration of furosemide (μM)
(B)
0.6
0.4
0.2
0
$r_{Bucolome}$
0
1
2
Unbound concentration of bucolome (μM)

Figure 2.

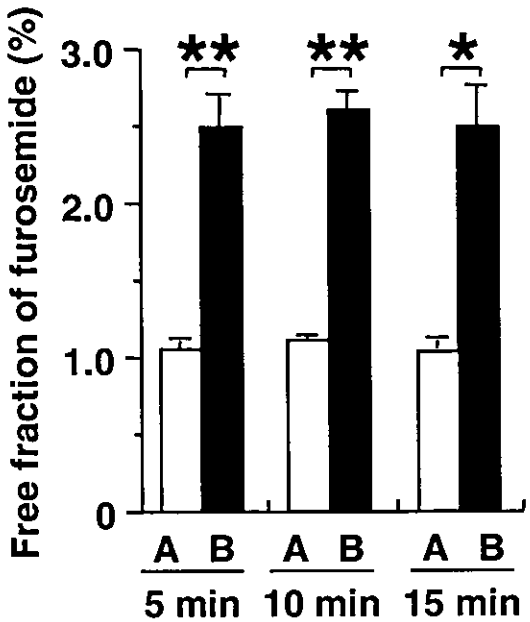

Free fraction of furosemide (%)
3.0
2.0
1.0
0
**
**
*
A B
A B
A B
5 min
10 min
15 min

Figure 3.

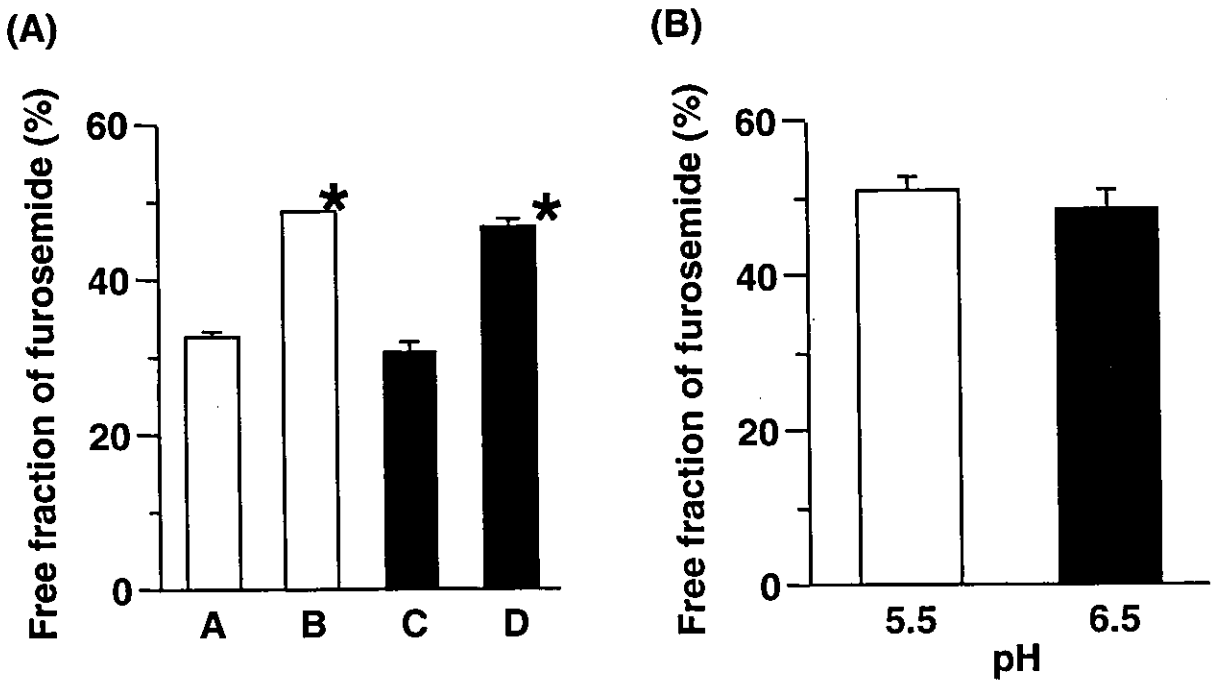
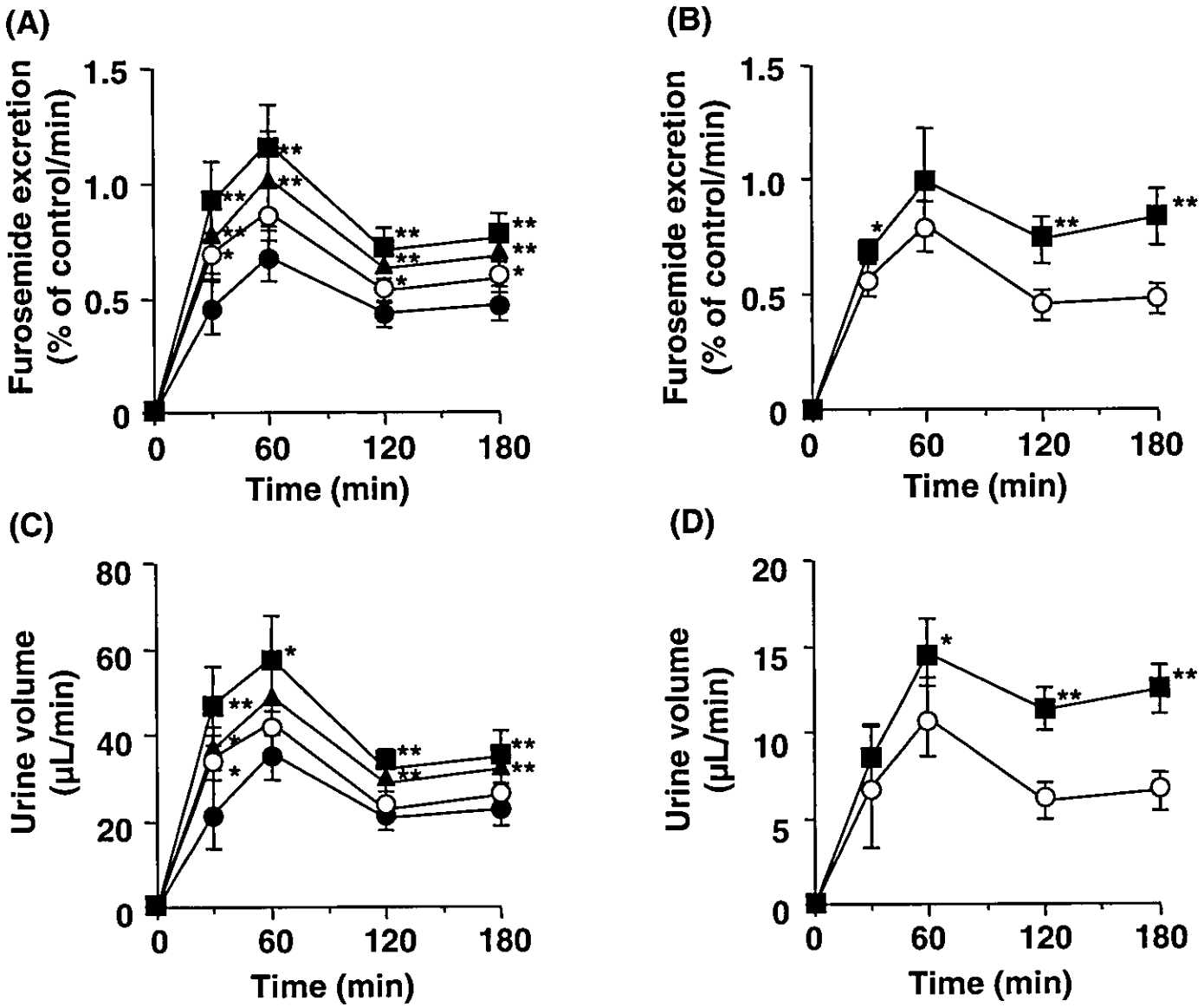

(A)
Free fraction of furosemide (%)
60
40
20
0
A
B
C
D
*
*
(B)
Free fraction of furosemide (%)
60
40
20
0
5.5
6.5
pH

Figure 4.


(A)
Furosemide excretion (% of control/min)
1.5
1.0
0.5
0
0
60
120
180
Time (min)
(B)
Furosemide excretion (% of control/min)
1.5
1.0
0.5
0
0
60
120
180
Time (min)
(C)
Urine volume (μL/min)
80
60
40
20
0
0
60
120
180
Time (min)
(D)
Urine volume (μL/min)
20
15
10
5
0
0
60
120
180
Time (min)

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Title page

Mutations in hURAT1 gene in pre-secretory reabsorption defect type of familial renal hypouricemia

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Running title: hURAT1 mutation in renal hypouricemia

Key words: hURAT1, mutation, renal hypouricemia, loss-of-function

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Abstract

To date, 11 loss-of-function mutations in human urate transporter 1 (hURAT1) gene have been identified in subjects with idiopathic renal hypouricemia. In the present studies, we investigated the clinical features and the mutations in hURAT1 gene in 7 families with pre-secretory reabsorption defect type renal hypouricemia and in one family with post-secretory reabsorption defect type. Twelve affected subjects and 26 family members were investigated. Mutations were analyzed by PCR and direct-sequencing method. Urate transporting activities of wild type and mutant hURAT1 were determined by ^{14}C-urate uptake in *Xenopus* oocytes. Mutational analysis revealed 3 previously reported mutations (G774A, A1145T, and 1639-1643 del-GTCCT) and a novel mutation (T1253G) in families with pre-secretory reabsorption defect type. Neither mutations in the coding region of hURAT1 gene nor significant segregation pattern of hURAT1 locus were detected in the post-secretory reabsorption defect type. All hURAT1 mutants had significantly reduced urate transporting activities than wild type ($P < 0.05$, n = 12), suggesting that T1253G is a loss-of-function mutation and that hURAT1 is responsible for the pre-secretory reabsorption defect type of familial renal hypouricemia. Future studies are needed to identify a responsible gene for the post-secretory reabsorption defect type of familial renal hypouricemia.