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Correlation between decrease of 11β-hydroxysteroid dehydrogenase activity and hypokalemia induced by furosemide in rats[1]

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KEY WORDS 11β-hydroxysteroid dehydrogenase; hypertension; hypokalemia

ABSTRACT

AIM: To investigate the correlation between decrease of 11β-hydroxysteroid dehydrogenase (11β-HSD) activity and hypokalemia induced by furosemide (Fur) in rats. **METHODS**: SD rats were given single dose or successive doses of Fur by gavage. The activity of 11β-HSD was evaluated by measuring the ratio of 11-dehydrocorticosterone (A) and corticosterone (B) in urine and conversion rate of B to A in renal cortex microsome preparation was determined with HPLC. **RESULTS**: After giving single dose of Fur (40, 100, and 250 mg/kg) or multiple doses of Fur (10, 20, and 100 mg/kg, bid × 20 d), the ratio of A/B was reduced by 29.0 %, 58.6 %, and 60.9 % at 0-2 h; 14.4 %, 36.0 %, and 44.9 %, respectively; the conversion rate of B to A was decreased by 29 %, 33 %, and 37 %; 6 %, 17 %, and 23 %, respectively. The serum potassium was significantly reduced by multiple doses of Fur (20 and 100 mg/kg, bid × 20 d) ($P < 0.01$). The reduction in serum potassium was positively correlated with the lowering of A/B ratio and the conversion of B to A ($P < 0.01$). **CONCLUSION**: The inhibition of renal 11β-HSD activity may be another new biochemical mechanism for hypokalemia induced by Fur.

INTRODUCTION

The 11β-hydroxysteroid dehydrogenase (11β-HSD) interconverts cortisol and cortisone in man, or corticosterone (B) and 11-dehydrocorticosterone (A) in rodents[1]. Two distinctly related congeners have been isolated, and conserved domains identified by multiple alignment and hydrophobic cluster analysis[1]. 11β-HSD1 is expressed in most tissues (hepatic, renal, brain, heart, and blood vessel) and it is NADP-dependent. It has low affinity to cortisol and usually catalyses reduction of cortisone to cortisol[2]. In contrast, 11β-HSD2 expressed in a limited range of tissues (placenta and kidney, especially in distal tubules) has high affinity to cortisol and is NAD-dependent. It catalyses dehydrogenation of cortisol to cortisone *in vivo* and is responsible for protecting mineralocorticoid receptors from inappropriate activation by cortisol[3,4]. The congenital deficiency of 11β-HSD2 induced hypertension and hypokalemia. This disorder was called "apparent mineralocorticoid excess" (AME).

Several endobiotics and xenobiotics have been found to modulate the activity of 11β-HSD[5,6] and caused excess mineralocorticoid effect and hypokalemia. Furosemide (Fur), an intensive diuretic, can increase renal tubular Na^+ reabsorption, which limits the natriuretic response and causes hypokalemia during chronic furosemide treatment[7]. Our previous study showed that Fur inhibited 11β-HSD activity in guinea pig kidney[5]. The later study suggested that Fur was an inhibitor of 11β-HSD[8,9]. In the present report, we evaluated the correlation between Fur on rat urinary A/B ratio and conversion rate of corticosterone in kidney cortex microsomes with hypokalemia, and explored the possible new mechanism of hypokalemia induced by Fur.

MATERIALS AND METHODS

Drugs and reagents Corticosterone (B), 11-dehydrocorticosterone (A), furosemide (Fur), glycyrrhizic acid (GA), and internal standard cortisol (F) were purchased from Sigma (St Louis, MO, USA). All other reagents used were of AR grade. Fur were dissolved in little dimethylsulfoxide and diluted with distilled water. GA was dissolved in distilled water.

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Corticosterone, 11-dehydrocorticosterone, and cortisol were dissolved in methanol and kept at −4 ℃.

Experimental animals Male SD rats, weighing 232 g ± 24 g (Grade Ⅱ, Certificate № 97001) were kept in room temperature (18 − 20 ℃) and fasted 12 h over night, but with water *ad libitum*. Next morning, rats were separated into five groups (six rats per group). Fur and GA were diluted with 0.9 % NaCl to a proper concentration. Then rats were administered 0.9 % NaCl, Fur 40, 100, and 250 mg/kg, and GA 250 mg/kg by gavage respectively. Animals were kept in metabolic cages for collecting urine at the period of 0 − 2 h and 2 − 6 h. After urine collection, rats were executed and the kidney cortex was separated to prepare microsomes.

In long-term experiment, male SD rats were kept at room temperature and maintained on a normal chow diet with free access to water. Rats were treated with Fur 10, 20, and 100 mg/kg (bid × 20 d, ig), 0.9 % NaCl and GA were taken as control. Urine (0 − 6 h) was collected from metabolic cages at 1, 5, 10, 15, and 20 d after medication. On the 20th day after collecting urine, rats were executed and the kidney cortex was separated to prepare microsomes. The blood samples were collected to assay serum potassium, sodium, and chlorine.

Microsome enzyme preparation Kidney cortex was obtained from rat received single or multiple doses of drugs. Tissue was scissored to strips and put in 10 mL test tube then washed twice by 1.15 % KCl solution. Phosphate-sucrose buffer (0.01 mol/L, pH 7.0) 6 mL was added into each tube and tissue was homogenized by a Tekmar Tissuemizer (Cincinnati, OH, USA) at 4 ℃. The homogenate was centrifuged at 10 000 × *g* for 30 min, then supernatant was centrifuged at 105 000 × *g* for 1 h. The microsomal pellet was resuspended in phosphate-sucrose buffer 0.01 mol/L (pH 7.0). Protein concentrations were measured by the Follin-Lowry method and the preparation was diluted to a concentration of 2 g/L prior to storage at −70 ℃.

Assay of conversion rate of corticosterone to dehydrocorticosterone The assays were performed as previously described[5]. Activity of 11β-HSD2 was determined by measuring the rate of conversion of corticosterone to 11-dehydrocorticosterone in the presence of NAD^+. Five minutes before incubation, 2 μL of Triton DF-18 was added to each milliliter of the microsome suspension. The mixture contained 20 μL of corticosterone 25 mg/L, 200 μL of NAD^+ 5 mmol/L, 200 μL (400 μg) of microsome suspension, and 380 μL Krebs-Henseleit buffer (pH 7.2). Samples were incubated in duplicate for 1 h at 37 ℃, the reaction was stopped on ice and 20 μL of cortisol 25 g/L was added as internal standard. The steroids were extracted with 3 mL methylene chloride by vortexing for 1 min then centrifuged at 640 × *g* for 15 min. The aqueous layer was thrown away and NaOH (0.1 mol/L) 0.3 mL was added to the organic phase, then the samples were mixed for 30 s and centrifuged at 640 × *g* for 10 min. The organic phase was transferred into a clean tube and evaporated under air stream at 45 ℃. The residue was dissolved in 150 μL of methanol, and 20 μL was injected into the HPLC apparatus.

The HPLC apparatus consisted of a model Shimadzu LC-10AD solvent delivery system, SPD-10A ultraviolet detector, and C-R2AX integraph. The mobile phase contained methanol-water (30:70, v/v) at a flow rate of 1.0 mL/min. The stainless steel Zorbax ODS column (250 mm × 4.6 mm, ID 4 μm) was kept at 30 ℃. The detector wave was UV 254 nm. The retention time for cortisol, corticosterone, and dehydrocorticosterone were 20, 24, and 32 min respectively (Fig 1).

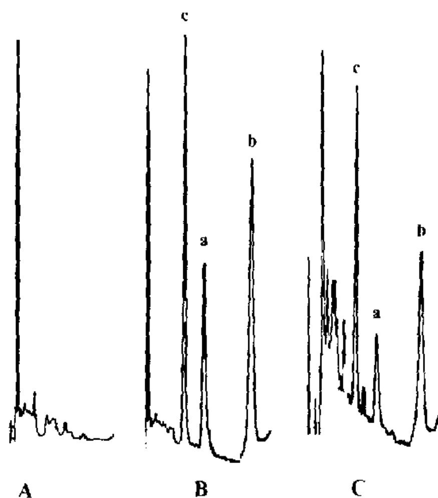

Fig 1. HPLC chromatograms of dehydrocorticosterone and corticosterone in rat urine. A: blank water; B: water spiked with dehydrocorticosterone 25 μg/L, corticosterone 25 μg/L, and internal standard; C: urine sample; a: dehydrocorticosterone; b: corticosterone; c: internal standard.

Statistical analysis Data were expressed as $\bar{x} \pm s$. The significance of differences was assessed by unpaired *t*-test.

RESULTS

Inhibitory effect of a single dose of Fur on renal 11β-HSD activity The ratio of dehydrocorticosterone to corticosterone (A/B) in rat urine was measured to assess the inhibitory effect of Fur on 11β-HSD and it was markedly decreased 2 h after medication in all groups of Fur (40, 100, and 250 mg/kg) ($P<0.05$ *vs* normal saline), but only 250 mg/kg group displayed a significant inhibitory effect during 2 – 6 h after medication ($P<0.05$ *vs* normal saline). The inhibitory effect was presented in a dose-dependent manner (Fig 2).

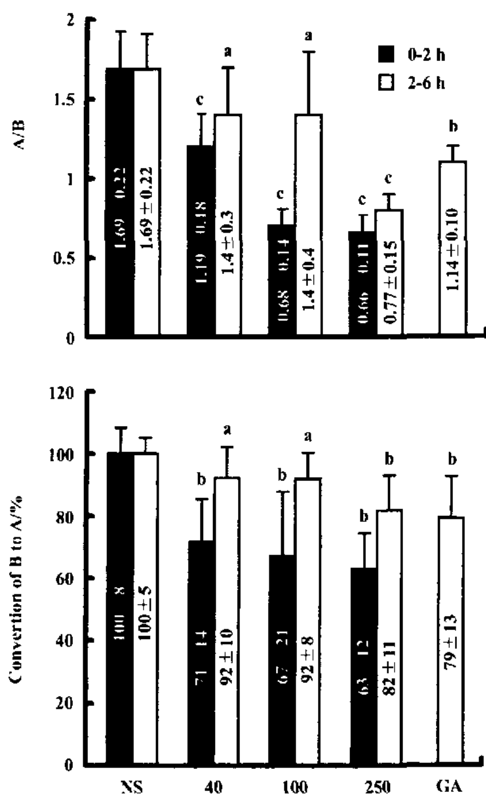

Fig 2. Inhibitory effect of furosemide (40, 100, and 250 mg/kg) on the ratio of dehydrocorticosterone to corticosterone (A/B) in rat urine and the percent conversion of corticosterone to dehydrocorticosterone (B to A) in rat renal cortex microsome preparation. (0 – 2 h): 2 h after administration; (2 – 6 h): 2 – 6 h after medication; GA: glycyrrhizic 250 mg/kg. $n=6$. $\bar{x}\pm s$. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* normal saline (NS).

A similar result was obtained in the microsome experiment. The percent conversion of corticosterone to dehydrocorticosterone by 11β-HSD2 was decreased by Fur (40, 100, and 250 mg/kg) ($P<0.05$) of 0 – 2 h after medication, and only 250 mg/kg inhibited the activity of 11β-HSD at 2 – 6 h (Fig 2).

The ratios of A/B in urine and conversion of B to A in microsome by 11β-HSD2 were reduced by 32.5 % and 21 % in GA group compared with normal saline group ($P<0.05$).

The total contents of corticosterone and dehydrocorticosterone in rat urine of saline and Fur (40, 100, and 250 mg/kg) group were (131 ± 133) ng, (360 ± 111) ng, (845 ± 352) ng, and (1016 ± 342) ng, respectively. It showed that the urine glucocorticoid and its metabolite in Fur group were far more than those in saline group ($P<0.01$). The content of corticosterone plus dehydrocorticosterone in GA group was (143 ± 109) ng and was not significantly different from saline group ($P>0.05$).

Inhibitory effect of Fur on renal 11β-HSD activity in successive administration The notable decrease in the value of urine A/B ratio was obtained when Fur and GA were given for consecutive 20 d medication ($P<0.05$ *vs* before medication) with the exception of Fur 10 mg/kg group. These phenomena had not been seen in normal saline group (Fig 3).

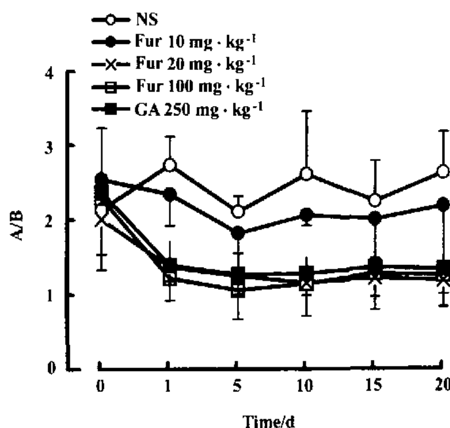

Fig 3. Effects of furosemide successive doses (10, 20, and 100 mg/kg, bid × 20 d, ig) on the ratio of dehydrocorticosterone to corticosterone (A/B) in rat urine. $n=6$. $\bar{x}\pm s$.

After Fur (20, 100 mg/kg) successive dosages for 20 d, the conversion of B to A was markedly reduced by 17 % and 23 %. GA made an 11 % reduction in the activity of renal 11β-HSD (Fig 4).

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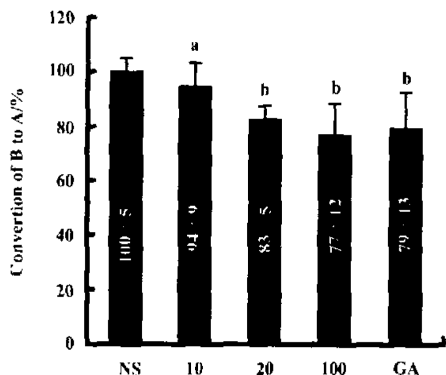

Fig 4. Effects of furosemide (10, 20, and 100 mg/kg) on the conversion of B to A in renal cortex microsome preparation after successive medication (bid × 20 d). GA: glycyrrhizica acid 250 mg/kg. $n = 6$. $\bar{x} \pm s$. [a]$P > 0.05$, [b]$P < 0.05$ *vs* NS.

Effect of Fur on serum electrolytes Potassium levels in the serum showed a slight decrease in each single dose group of Fur, but there was no significant difference compared with normal saline group ($P < 0.05$). The sodium and chlorine levels in serum were significantly decreased ($P < 0.05$ or 0.01) (Tab 1). In consecutive medication test, potassium levels in serum were reduced after giving Fur (20, 100 mg/kg, bid × 20 d) ($P < 0.01$ *vs* normal saline). But sodium levels in serum were significantly increased in Fur 100 mg/kg group ($P < 0.05$ *vs* normal saline). The reduction of serum potassium positively correlated with the lowering of urine A/B ratio and the conversion rate of B to A in microsome preparation, the correlation coefficient (r value) was 0.9994 and 0.9991, respectively (Fig 5). These results indicated that the fall in plasma potassium was related to the decline of 11β-HSD2 activity.

Tab 1. Effects of single or multiple dose of furosemide and glycyrrhizic acid (GA) 250 mg/kg on rat serum electrolyte. $n = 6$. $\bar{x} \pm s$. [a]$P > 0.05$, [b]$P < 0.05$, [c]$P < 0.01$ *vs* NS.

Furosemide/ mg·kg^{-1}	K^+/mmol·L^{-1}	Na^+/mmol·L^{-1}	Cl^-/mmol·L^{-1}
Single-dose			
NS	7.1 ± 0.9	142.5 ± 1.9	107 ± 4
40	6.8 ± 1.2[a]	140.4 ± 2.8[a]	102.6 ± 2.5[b]
100	6.6 ± 1.2	139.6 ± 2.6[b]	98.3 ± 2.2[c]
250	6.6 ± 0.6[a]	137.9 ± 2.4[c]	95 ± 3[c]
GA	6.5 ± 1.0[a]	177 ± 24[c]	138 ± 19[c]
Multi-dose			
NS	6.8 ± 0.5	140.1 ± 1.7	107.2 ± 1.8
10	6.6 ± 1.4[a]	142 ± 4[a]	106 ± 3[a]
20	5.4 ± 0.6[c]	142 ± 3[a]	103 ± 3[c]
100	4.6 ± 0.7[c]	144 ± 4[b]	102 ± 6[c]

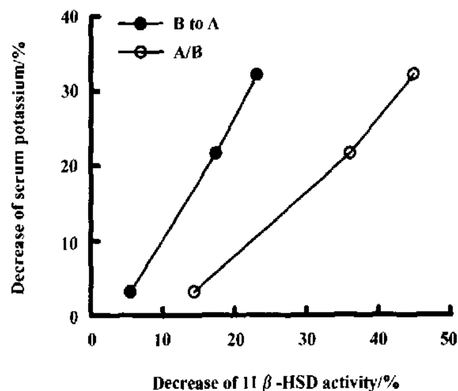

Fig 5. Correlation between the decrease of serum potassium and the inhibition of renal 11β-HSD activity after successive medication of furosemide (10, 20, and 100 mg/kg, bid × 20 d) by gavage. The decrease of the conversion of B to A in renal cortex microsome preparation (B to A) and the ratio of A/B in urine were positively correlated with reduction of serum potassium. ($r = 0.9991$ for B to A; $r = 0.9994$ for A/B).

DISCUSSION

In normal condition, the glucocorticosteroid level was higher than aldosterone in kidney and mineralocorticoid receptor had equal affinity to aldosterone and cortisol or corticosterone. 11β-HSD protected renal from corticosterone intoxication by converting corticosterone to its inactive metabolite dehydrocorticosterone. Because aldosterone was not metabolized by the 11β-HSD enzyme, it normally had unimpeded access to the mineralocorticoid receptor. In congenital 11β-HSD deficiency (AME) and after administration of the 11β-HSD inhibitor, the protective mechanism failed, intrarenal corticosterone level rose, and corticosterone gained inappropriate access to mineralocorticoid receptors resulting in hypokalemia and sodium retention[10]. Our previous investigation showed that furosemide was a single potent inhibitor of 11β-HSD in all diuretics. In the present study, we found that the lowering of the conversion rate from B to A, the declining of urinary A/B ratio, and the decrease in the level of serum potassium revealed a dose-dependent profile when Fur was medicated in a single or successive doses in intact rats.

The most important thing was that the reduction in serum potassium was positively correlated with the decrease of urinary A/B ratios and the percent conversion of B to A by cortex microsome.

It is known that Fur can induce hypokalemia especially in a long period treatment or at high doses. This is attributed to the increase in delivery of sodium to the distal tubule, so that a large fraction of sodium is available for exchange with potassium. Our results showed that the hypokalemia of rat was presented in a dose-dependent manner after successive medication of Fur (10, 20, 100 mg/kg, bid × 20 d). Opposed to a single dose experiment, the sodium level in serum was increased ($P < 0.05$) in Fur (100 mg/kg, bid × 20 d). Therefore, the inhibition of 11β-HSD may be another important basic biochemistry mechanism to account for the increase in loss of potassium in urine treated with Fur.

The terminal half-life was found to be 29 min for the 10 mg/kg group and 49 min for the 40 mg/kg group in rat[11]. It showed that metabolism of furosemide was saturable. We found that the activity of 11β-HSD was inhibited after 2 h medication in all groups of Fur (40, 100, and 250 mg/kg), but the plasma concentration of Fur (40, 100 mg/kg) might be lower than effective inhibitory concentration, and only 250 mg/kg group displayed a significant inhibitory effect in 2 – 6 h after medication. These results indicated that the inhibitory effect of furosemide on 11β-HSD was time-dependent.

The total content of corticosterone and dehydrocorticosterone found in the urine in the Fur groups (40, 100, and 250 mg/kg) was 2.7, 6.4, and 7.7 times as high as in the saline group during 2 – 6 h after administration ($P < 0.01$). But this phenomenon was not seen in GA group. These results suggested that the increase of glucocorticoids and metabolites in urine was not related to the inhibition of 11β-HSD activity, but it might reflect that activation of hypothalamic-pituitary adrenal axis was caused by reduction of blood volume, which was induced by powerful diuretic action of Fur[12].

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速尿对大鼠肾 11β-羟基类固醇脱氢酶活性的抑制作用与其低血钾的相关性[1]

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关键词 11β-羟基类固醇脱氢酶；高血压；低钾血症

目的：研究速尿对大鼠 11β-羟基类固醇脱氢酶活性的抑制作用与低血钾的相关性．**方法**：SD 大鼠给予速尿 40，100 和 250 mg/kg 单次灌胃，或 10，20 和 100 mg/kg 连续灌胃 20 d，每天两次． HPLC 法测定尿中脱氢皮质酮(A)与皮质酮(B)的比率及肾皮质微

粒体酶对皮质酮的转化率评定肾 11β-羟基类固醇脱氢酶活性. **结果**: 速尿(40, 100, 250 mg/kg)单次或(10, 20, 100 mg/kg)连续灌胃, 大鼠尿中 A/B 分别降低了 29.0 %, 58.6 %, 60.9 % (0 - 2 h)和 14.4 %, 36.0 %, 44.9 %. 皮质酮的转化率降低了 29 %, 33 %, 37 %和 6 %, 17 %, 23 %, 速尿(20, 100 mg/kg)连续给药组血钾显著降低($P < 0.01$). 血钾的降低与皮质酮的转化率及尿中 A/B 值的变化成正相关. **结论**: 速尿对 11β-羟基类固醇脱氢酶活性的抑制可能是其引起低血钾的另一个重要的基本生化机制.

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