ภาคผนวก

Clinical Endocrinology (2011) 74, 152–159 doi: 10.1111/j.1365-2265.2010.03912.x

ORIGINAL ARTICLE

Aldosterone increases Na^+-K^+-ATPase activity in skeletal muscle of patients with Conn's syndrome

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Summary

Objective In Conn's syndrome, hypokalaemia normally results from renal potassium loss because of the effect of excess aldosterone on Na^+-K^+-ATPase in principal cells. Little is known about the effect of aldosterone on cellular potassium redistribution in skeletal muscle. Our study determined the effect of aldosterone on muscle Na^+-K^+-ATPase.

Design Muscle biopsies were taken from six patients immediately before and 1 month after adrenalectomy. Ten age-matched subjects with normal levels of circulating aldosterone served as controls.

Results Average plasma aldosterone was significantly higher in presurgery (235·0 ± 51·1 pg/ml) than postsurgery (64·5 ± 25·1 pg/ml) patients. Similarly, Na^+-K^+-ATPase activity, relative mRNA expression of α_2 (not α_1 or α_3) and β_1 (not β_2 or β_3), and protein abundance of α_2 and β_1 subunits were greater in pre- than postsurgery samples (128·7 ± 12·3 *vs* 79·4 ± 13·3 nmol·mg/protein/h, 2·45 ± 0·31 *vs* 1·04 ± 0·17, 1·92 ± 0·22 *vs*1·02 ± 0·14, 2·17 ± 0·33 *vs* 0·98 ± 0·09 and 1·70 ± 0·17 *vs* 0·90 ± 0·17, respectively, all $P < 0·05$). The activity and mRNA expression of the α_2 and β_1 subunits correlated well with plasma aldosterone levels ($r = 0·71$, $r = 0·75$ and $r = 0·78$, respectively, all $P < 0·01$).

Conclusions Our study provides the first evidence in human skeletal muscle that increased plasma aldosterone leads to increased Na^+-K^+-ATPase activity via increases in α_2 and β_1 subunit mRNAs and their protein expressions. The increased activity may contribute in part to the induction of hypokalaemia in patients with Conn's syndrome.

(Received 6 August 2010; returned for revision 23 August 2010; finally revised 27 September 2010; accepted 18 October 2010)

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Introduction

Na^+-K^+-ATPase, mediating the active transport of Na^+ and K^+ across the cell membrane of most living cells, is essential for maintaining resting membrane potential, cell volume and osmotic balance.[1] The enzyme is a heterodimeric molecule consisting of catalytic α and glycosylated β subunits.[2] Different species and tissues have different isoforms of Na^+-K^+-ATPase α and β subunits.[3,4] In mammals, the α subunit exists in at least four isoforms, α_1–α_4.[5] Subunit α_2 is the major catalytic isoform expressed in rodent skeletal muscle[6] and is also a prominent subunit in human skeletal muscle.[7] During exercise, the α_2 subunit translocates to the plasma membrane of human skeletal muscle[8] and it has been shown that insulin treatment in rats induces redistribution of the α_2 subunit to the plasma membrane as well.[9] Our previous study showed that thyroid hormone upregulates the human skeletal muscle Na^+-K^+-ATPase α_2 subunit.[10] In addition to the α subunit, there are at least 3 β isoforms, β_1–β_3,[5,11] which appear to be important for preservation of the stability of α subunits and for transportation of the mature enzyme complex to plasma membranes.[4,12]

Aldosterone, a mineralocorticoid hormone, is an important regulator of Na^+-K^+-ATPase gene expression in renal tubular and other polarized epithelial cells involved in Na^+ and K^+ ions transport.[13,14] In the kidney, aldosterone increases gene expression and activity of Na^+-K^+-ATPase in renal epithelial cells.[13,15] In patients with hyperaldosteronism, it is generally known that the major cause of hypokalaemia is renal potassium loss induced by the effect of aldosterone on the principal cells of collecting tubules. However, the mechanism of aldosterone action on cellular potassium redistribution is poorly understood. Knowledge of this mechanism will provide important information for further clinical therapeutic applications.

On one hand, Dorup *et al.*[16] reported that infusion of aldosterone in rat skeletal muscle cells for 7 days produces hypokalaemia and a graded reduction in K^+ content and concluded that mineralocorticoids induce a downregulation of Na^+-K^+-ATPase, which is secondary to a concomitant K^+ deficiency. On the other hand, Ikeda *et al.*[17] reported that aldosterone directly stimulates mRNA synthesis of Na^+-K^+-ATPase α_1 and β_1 subunits and protein accumulation in adult and neonatal rat ventricular cardiocytes.

In this study, we examined muscle biopsies of patients with Conn's syndrome who had an adenoma at the adrenal gland, producing high levels of plasma aldosterone and low plasma potassium. To rule out the effect of low plasma potassium on Na^+-K^+-ATPase activity, plasma potassium was corrected prior to the experiment, so that the direct effect of hyperaldosteronism could be examined. It was found that increased plasma aldosterone level leads to increased Na^+-K^+-ATPase activity via increases in α_2 and β_1 subunit mRNAs, as well as their protein expressions. All of these parameters were restored to normal levels following adrenalectomy.

Subjects and methods

Participants

Six patients, four women and two men, diagnosed with Conn's syndrome at Ramathibodi Hospital, Bangkok, Thailand, were enrolled in this study. Diagnosis of Conn's syndrome was based on symptoms and signs of clinical hyperaldosteronism including hypertension, hypokalaemic metabolic alkalosis and renal K^+ loss. Laboratory confirmations included high aldosterone/renin ratio (>20 when plasma aldosterone >160 pg/ml), nonsuppressible hyperaldosteronism and a positive imaging of an adenoma. Antihypertensive agents, nifedipine and/or hydralazine, were used to control blood pressure (Table 1). Hypokalaemia was corrected by oral potassium chloride solution (KCl in water, 20 mEq/10 ml). Hypertension and hypokalaemia were corrected at least 2 weeks before surgery (removal of adenoma). The plasma aldosterone level was also measured for at least 2 weeks after correction of hypertension and hypokalaemia. All participants were given dietary potassium, about 70 mEq/day, and sodium, 100 mEq/day, throughout the entire individual's study, and the dietary potassium and sodium intake of each patient was formally recorded by a nutritionist. All patients were treated with surgical excision of the affected adrenal glands. They discontinued antihypertensive agents and potassium chloride supplements within 2 weeks after surgery. There was no postsurgical medication except analgesics for 2 weeks. The control group was age-matched control subjects composed of seven women and three men undergoing elective knee or hip surgery with normal aldosterone levels. All participants gave written informed consent after receiving oral and written information concerning the study according to the Declaration of Helsinki II. The study protocol was approved by the ethical committee for research involving human subjects of Ramathibodi Hospital, Mahidol University, Thailand.

Methods

Blood samples of patients with Conn's syndrome were collected before surgery (and at least 2 weeks after correction of hypertension and hypokalaemia to avoid the effect of these factors on Na^+ – K^+ ATPase activity) and 1 month after surgical treatment. In the control group, blood samples were collected before their operation.

Table 1. Clinical features of the first presentation and comparison among pre- and postadrenalectomies of six patients with Conn's syndrome and ten controls

	Conn's syndrome			*P*-value		*P*-value	
Patients' characteristic	First presentation	Pre-surgery	Postsurgery	Pre. versus Post.	Control	Pre. versus Con.	Post. versus Con.
Age (year)		41·8 ± 7·8			40·9 ± 7·0	0·93	
Weight (kg)		60·8 ± 5·2			59·8 ± 6·7	0·74	
Body mass index (kg/m^2)		23·4 ± 0·5			23·2 ± 0·7	0·39	
Systolic blood pressure (mmHg)	184·1 ± 10·7	131·3 ± 5·5	131·2 ± 6·1	0·92	128·0 ± 4·5	0·24	0·3
Diastolic blood pressure (mmHg)	108·8 ± 6·6	78·7 ± 2·9	78·2 ± 3·9	0·72	75·0 ± 4·5	0·07	0·16
Serum sodium (mmol/l)	138·5 ± 3·0	139·5 ± 2·8	138·8 ± 1·7	0·46	139·0 ± 2·7	0·74	0·88
Serum potassium (mmol/l)	2·8 ± 0·29	3·95 ± 0·18	4·02 ± 0·13	0·34	4·04 ± 0·32	0·48	0·88
Serum chloride (mmol/l)	100·0 ± 2·3	105·8 ± 2·6	105·7 ± 3·6	0·86	106·8 ± 2·2	0·47	0·5
Serum bicarbonate (mmol/l)	32·2 ± 3·5	25·3 ± 1·4	24·0 ± 2·8	0·14	24·1 ± 2·5	0·22	0·94
Total potassium intake (mmol/l)		75·5 ± 12·7	69·3 ± 9·8	0·2	69·8 ± 10·5	0·38	0·93
Total sodium intake (mmol/l)		94·3 ± 9·8	98·2 ± 10·6	0·28	99·0 ± 11·1	0·4	0·88
24-h urine potassium (mmol/day)	65·2 ± 17·8	62·8 ± 12·2	55·7 ± 11·6	0·14	57·2 ± 11·9	0·39	0·8
24-hr urine sodium (mmol/day)	138·7 ± 19·3	83·2 ± 13·2	86·7 ± 13·6	0·4	89·0 ± 14·1	0·42	0·75
Plasma aldosterone level (N 10–160 pg/ml)		235·0 ± 51·1	64·5 ± 25·1	< 0·01	66·5 ± 13·0	< 0·01	0·86
Plasma renin activity (N 0·2–2·8 ng/ml/h)		0·17 ± 0·06					
Antihypertensive agents							
Nifedipine (mg/day)		18·3 ± 7·6 (range 10–30)					
Hydralazine (mg/day)		33·3 ± 30·3 (range 0–75)					

Pre. = presurgery, Post. = postsurgery, Con. = control, N = normal.

Plasma aldosterone was measured with a solid-phase radioimmunoassay (Coat-A-Count® Aldosterone kit; Diagnostic Products Corporation, Los Angeles, CA, USA). Plasma renin activity was measured with a radioimmunoassay using a REN-CT2 kit (Cisbio Bioassays, Bagnols-sur-Céze Cedex, France). Serum electrolyte, BUN and creatinine levels were determined using a Technicon Auto Analyzer (Pulse Instrumentation Ltd., Saskatchewan, Canada).

Muscle biopsy

To avoid the effect of hypertension and hypokalaemia on Na^+-K^+-ATPase, all patients were corrected for hypertension and hypokalaemia at least 2 weeks before muscle biopsies, which were taken immediately before and 1 month after removal of the affected adrenal glands. The surgical procedure was performed as previously described.[10] Vastus lateralis muscle samples were divided into three parts for the Na^+-K^+-ATPase activity assay, quantitative analysis of mRNA and immunoblotting of proteins. Muscle samples were frozen immediately in liquid nitrogen and stored at −80 °C until assayed. In control subjects, vastus lateralis muscle samples were obtained before elective knee or hip surgery and only samples that showed no sign of necrosis and were judged to be well perfused and oxygenated were used.

Na^+-K^+-ATPase activity

K^+-stimulated 3-*O*-methylfluorescein phosphatase assay (3-*O*-MFPase) was used to measure skeletal muscle Na^+-K^+-ATPase activity as previously described[18] with some minor modifications. A muscle sample (50 mg) was homogenized in 450 μl of homogenate buffer (30 mM histidine (pH 7·4), 2 mM EDTA and 250 mM sucrose), freeze-thawed four times and then diluted 1:10 in cold homogenate buffer. A 15-μl aliquot of this homogenate was added to an assay solution containing 19·5 μM 3-*O*-methyfluorescein phosphate, 4 mM $MgCl_2$, 1·25 mM EDTA and 80 mM Tris (pH 7·6). After 90 s, a 10-μl aliquot of 2·58 M KCl (final concentration 10 mM) was added. Activity was calculated from the difference in the slope of fluorescence before and after the addition of KCl. The excitation wavelength was set at 475 nm and emission wavelength at 515 nm, with 5-nm slit width. Muscle homogenate protein content was determined using a BCA Assay kit (Pierce, Rockford, IL, USA). Average specific 3-*O*-MFPase activity was determined from three experiments, and intra- and inter-assay variability were approximately 9% and 5·8%, respectively.

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described.[10] Specific primers and probes were used to amplify mRNA of Na^+-K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 subunits.[10,19] Probes were 5′- and 3′-labelled with 6-carboxyfluorescein and 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine, respectively. GAPDH mRNA content (as internal control) was determined by using commercial primers and probes (P/N 4326317E; Applied Biosystems, Carlsbad, CA, USA). Primer and probe optimization and validation were carried out. The relative efficiency of the test targets of all six subunit isoforms of Na^+-K^+-ATPase was calibrated. Thermal cycling (ABI 7000 real-time PCR system) was conducted as follows: incubation at 50 °C for 2 min; 95 °C for 10 min; and 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 s. Each experiment was conducted in triplicate. The intra- and inter-assay coefficients of variance for each gene were 8 and 9% for α_1, 9 and 8% for α_2, 10 and 8% for α_3, 9 and 7% for β_1, 11 and 9% for β_2, and 10 and 11% for β_3, respectively. The comparative C_T method (multiplex PCR, same tube) was used to calculate the relative mRNA level (Applied Biosystems). There was no statistical difference of the average C_T of GAPDH mRNA among the samples ($P = 0{\cdot}13$).

Immunoblot analysis

Immunoblotting was performed as previously described.[10] In brief, a muscle sample (30 mg) was homogenized on ice in a buffer. Twenty μg (for analysis of $\beta_{1\text{-}3}$) or 40 μg (for analysis of $\alpha_{1\text{-}3}$) of protein was separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was incubated for 2 h with a blocking buffer (5% nonfat milk in Tris-buffered saline–Tween 20 (TBST)) and then incubated overnight at 4 °C in primary antibodies (anti-α_1 polyclonal antibody (lot no 06520), anti-α_2 polyclonal antibody (lot no AB9094), anti-β_1 polyclonal antibody (lot no 06170), anti-β_2 polyclonal antibody (lot no 610993; Upstate Biotechnology, Lake Placid, NY, USA), anti-α_3 monoclonal antibody (lot no MA3-915; Affinity Bioreagents, Golden, CO, USA) and anti-β_3 monoclonal antibody (lot no 610993; Transduction Laboratories, Franklin Lakes, NJ, USA)). Anti-α (α_1–α_3) and anti-β (β_1–β_3) antibodies were diluted 1:2000 and 1:5000, respectively. Membranes were washed in 0·05% TBST and incubated for 1 h in horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse for monoclonal antibody or goat anti-rabbit immunoglobulins for polyclonal antibody, diluted 1: 10 000 in TBST). After three washes, membranes were incubated with chemiluminescent substrate (Pierce SuperSignal, West Pico, IL, USA). The signal was detected and recorded (Pierce CL-X Posure, West Pico, IL, USA). Resulting autoradiographs were densitometrically scanned and quantified.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data were reported as mean ± SD. Relative mRNA and protein expression were reported as mean ± SE. Comparisons were made using two-tailed paired (within group) and unpaired *t*-test (between groups). Correlation was determined by linear regression. There was significant difference if P value < 0·05.

Results

Clinical features and laboratory data of six patients with Conn's syndrome at the first time of diagnosis are shown in Table 1. As expected, blood pressures and HCO_3^- levels of patients with Conn's syndrome were higher than those of normal controls. Hypokalaemia was also observed. These parameters were corrected prior to

biopsy. As shown in Table 1, there are no significant differences in blood pressure, serum potassium level, acid–base, and sodium and potassium balance among pre-adrenalectomy patients, postadrenalectomy patients and control subjects. In contrast, in patients with Conn's syndrome, the average presurgery plasma aldosterone level (235·0 ± 51·1 pg/ml) was approximately 3·5 times higher than that of postsurgery (64·5 ± 25·1 pg/ml) and of a normal control group (66·5 ± 13·0 pg/ml) ($P < 0·01$). There was no significant difference in plasma aldosterone level between postsurgery patients and control subjects (Table 1 and Fig. 1a).

The average skeletal muscle Na^+-K^+-ATPase activity of presurgery Conn's syndrome patients (128·7 ± 12·3 nmol·mg/protein/h) was about 1·6 and 1·5 times higher than that of postsurgery (79·4 ± 13·3 nmol·mg/protein/h) and control subjects (88·7 ± 5·1 nmol·mg/protein/h), respectively ($P < 0·05$) (Fig. 1b). There was no difference in Na^+-K^+-ATPase activity between postsurgery patients and control subjects. A positive correlation existed between the plasma aldosterone level and Na^+-K^+-ATPase activity of patients with Conn's syndrome and control subjects ($r = 0·71$, $P < 0·01$), (Fig. 2a), which implied that the increased muscle Na^+-K^+-ATPase activity in Conn's syndrome may have been upregulated by aldosterone.

In the skeletal muscle of patients with Conn's syndrome, mRNA expressions of Na^+-K^+-ATPase α_2 and β_1 subunits were higher in presurgery (hyperaldosteronism) than in postsurgery (normoaldosteronism) biopsy samples by about 2·4 (2·45 ± 0·31 *vs* 1·04 ± 0·17, $P < 0·05$, for α_2) and 1·9 (1·92 ± 0·22 *vs* 1·02 ± 0·14, $P < 0·05$, for β_1) times, respectively (Fig. 3). In contrast, there were no differences in α_2 and β_1 subunit expressions between postsurgery and control subjects. There were no changes in α_1, α_3, β_2 or β_3 subunit mRNA levels among the three groups.

Elevation of α_2 and β_1 subunit mRNA expression levels resulted in a corresponding increase in Na^+-K^+-ATPase α_2 and β_1 subunit proteins about 2·2 (2·17 ± 0·33 *vs* 0·98 ± 0·09, $P < 0·05$, for α_2) and 1·8 (1·70 ± 0·17 *vs* 0·90 ± 0·17, $P < 0·05$, for β_1) times, respectively, in presurgery compared to postsurgery (Fig. 4). As expected, there was no change in protein levels of α_1, α_3, β_2 or β_3 subunits among the three groups (Fig. 4).

In addition, the expression levels of Na^+-K^+-ATPase α_2 and β_1 subunit mRNA were positively correlated with plasma aldosterone

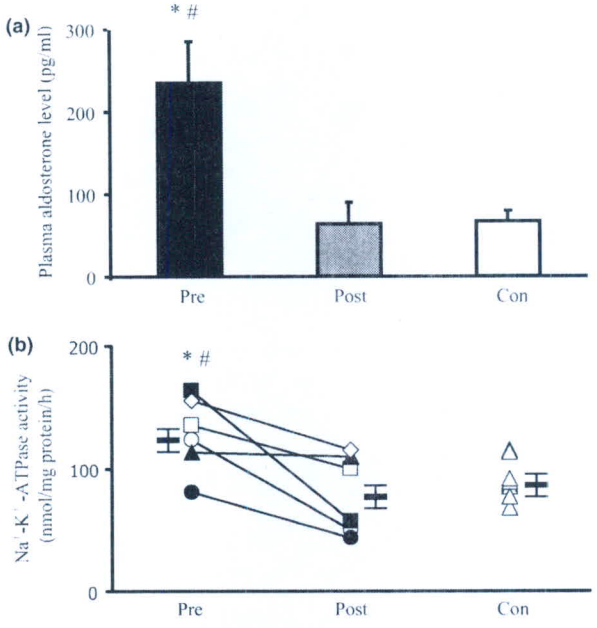

Fig. 1 (a) Plasma aldosterone level in patients with Conn's syndrome and control subjects. Data are mean ± SD (bar); $n = 6$ for Conn's syndrome patients, $n = 10$ for control subjects. Pre, presurgery; Post, postsurgery; Con, control. $^*P < 0.05$ compared with postsurgery group, $\#P < 0.05$ compared with control group. (b) Na^+-K^+-ATPase activity in the skeletal muscle of individual patients with Conn's syndrome and control subjects. All symbols joined by lines indicate individual responses, while nearby symbols indicate group average as mean ± SD. Each symbol represents each patient, △ = control subject.

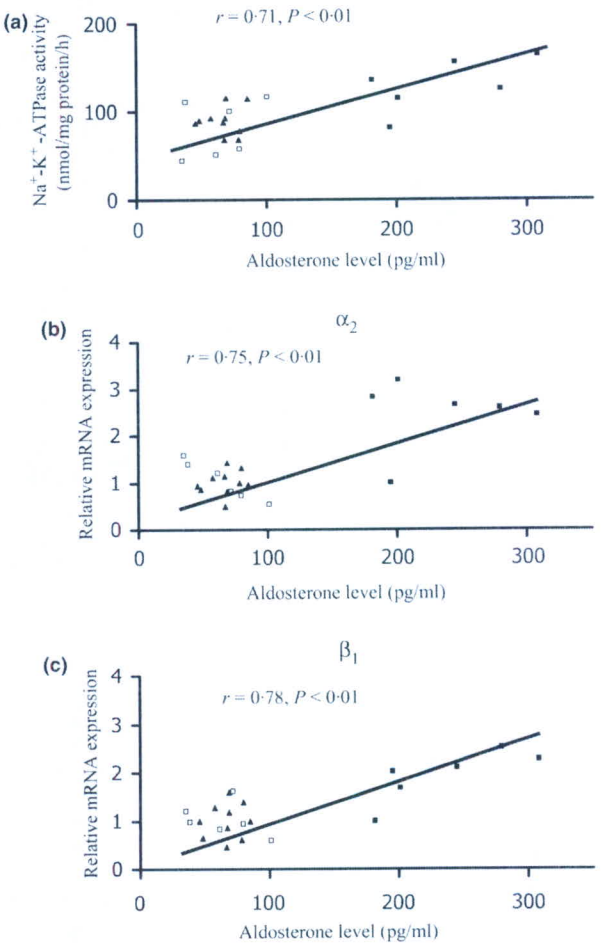

Fig. 2 Correlation between skeletal muscle Na^+-K^+-ATPase activity (a), α_2 isoform mRNA (b) and β_1 isoform mRNA level (c) with plasma aldosterone level in presurgery ($N = 6$), postsurgery patients ($N = 6$) and control subjects ($N = 10$). Correlation was determined by linear regression (r value). ■ = presurgery Conn's syndrome patient. □ = postsurgery Conn's syndrome patient. ▲ = control subject.

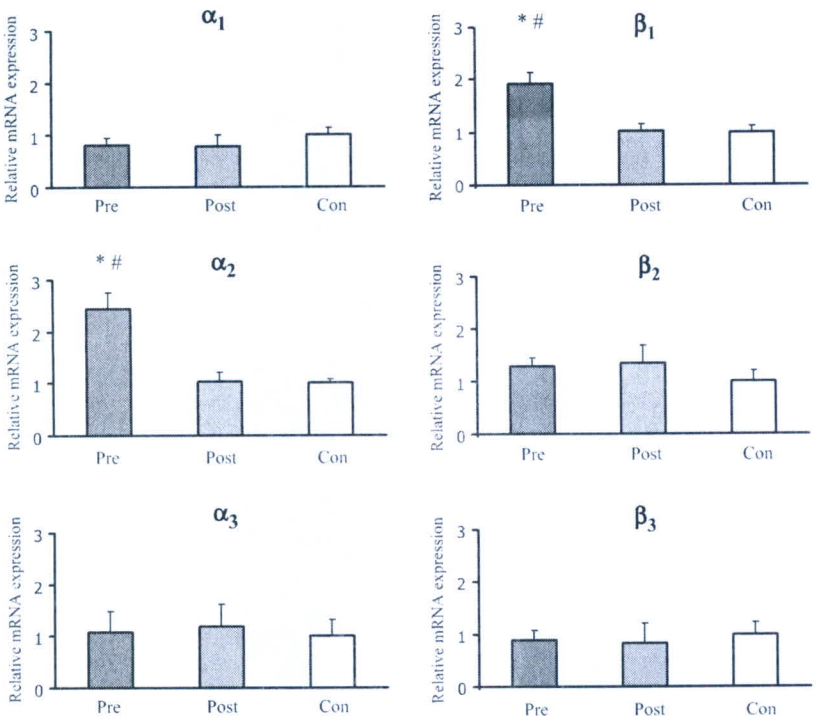

Fig. 3 Relative mRNA level of the Na^+-K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms in skeletal muscles of pre- and postsurgery patients and control subjects. Na^+-K^+-ATPase isoform mRNA level is expressed relative to that of GAPDH, and the control level was set as 1 for comparison purposes. The value is shown as mean ± SE (bars). Pre, presurgery $N = 6$; Post, postsurgery $N = 6$; Con, control $N = 10$. *$P < 0.05$ compared with postsurgery patients, #$P < 0.05$ compared with controls.

levels ($r = 0{\cdot}75$, $P < 0{\cdot}01$ for α_2 and $r = 0{\cdot}78$, $P < 0{\cdot}01$ for β_1) (Fig. 2b,c).

Discussion

Hypertension, hypokalaemia and muscle weakness are classical presentations of Conn's syndrome. Hypokalaemia can cause muscle weakness. In general, hypokalaemia can result from potassium depletion induced by abnormal loss of potassium or by cellular potassium redistribution.[20] The aetiology of hypokalaemia varies and is based on clinical setting and laboratory data.[20]

Aldosterone is a critical hormone in renal handing of potassium by stimulating Na^+-K^+-ATPase synthesis and activity in the collecting tubules. Moreover, the role of extrarenal mineralocorticoid in regulating potassium excretion via the gastrointestinal (GI) tract and potassium redistribution in many cell types has been reported.[21,22] Human skeletal muscle comprises nearly 60% of the lean body mass and contains the largest store of the body's potassium as well as a major portion of Na^+-K^+-ATPase. Thus, aldosterone might also play an important role in modulating cellular redistribution of potassium via Na^+-K^+-ATPase activity.

Although it has been generally assumed that the regulation of Na^+-K^+-ATPase by aldosterone observed in rodent skeletal muscle or cell culture is likely to be similar to that in human skeletal muscle, this has not been shown directly in human skeletal tissue, especially in pathological conditions. This study demonstrated for the first time in human skeletal muscle that Na^+-K^+-ATPase activity is modulated by high plasma aldosterone levels. An increase in Na^+-K^+-ATPase activity in hyperaldosterone (Conn's syndrome) patients was likely because of an increase in the levels of α_2 and β_1 (but not α_1, α_3, β_2 or β_3) subunit mRNA and protein, and these parameters returned to normal levels after surgical removal of the adenoma. It is unlikely that an increase in activity and gene expression of Na^+-K^+-ATPase was as a result of an increase in the blood pressure, low plasma potassium level or acid–base disturbance as both pre- and postadrenalectomy groups had these parameters comparable to those of the control group. In addition, it is unlikely that the changes observed were because of the use of antihypertensive agents as both nifedipine and hydralazine have been shown to have no effect on Na^+-K^+-ATPase subunits in the cardiac muscles of rats,[23] and nifedipine has no effect on Na^+-K^+-ATPase activity in isolate smooth muscle membranes from rat aorta.[24] However, we cannot totally exclude the possibility that these drugs might have some effects on skeletal muscle Na^+-K^+-ATPase activity. Unfortunately, we could not obtain the appropriate controls (muscle biopsies from hypertensive patients before and after using the same hypertensive drugs and doses), because of ethical issues, to clarify this confounding factor.

The correlation of the plasma aldosterone level with both mRNA and protein abundance of the α_2 and β_1 subunits implies that increased plasma aldosterone leads to the upregulation of Na^+-K^+-ATPase protein expression, at least in part, at the transcriptional level. These results are in contrast with those of Dorup *et al.*[16] who reported that administration of aldosterone into a rat for 7 days produces hypokalaemia and a graded reduction in K^+ content in skeletal muscle, which closely correlates with a downregulation of the [^{3}H] ouabain-binding sites, and concluded that mineralocorticoids induce a downregulation of Na^+-K^+-ATPase, which is secondary to the concomitant K^+ deficiency. It is well known that hypokalaemia has a major effect on the Na^+-K^+-ATPase. *In vivo*

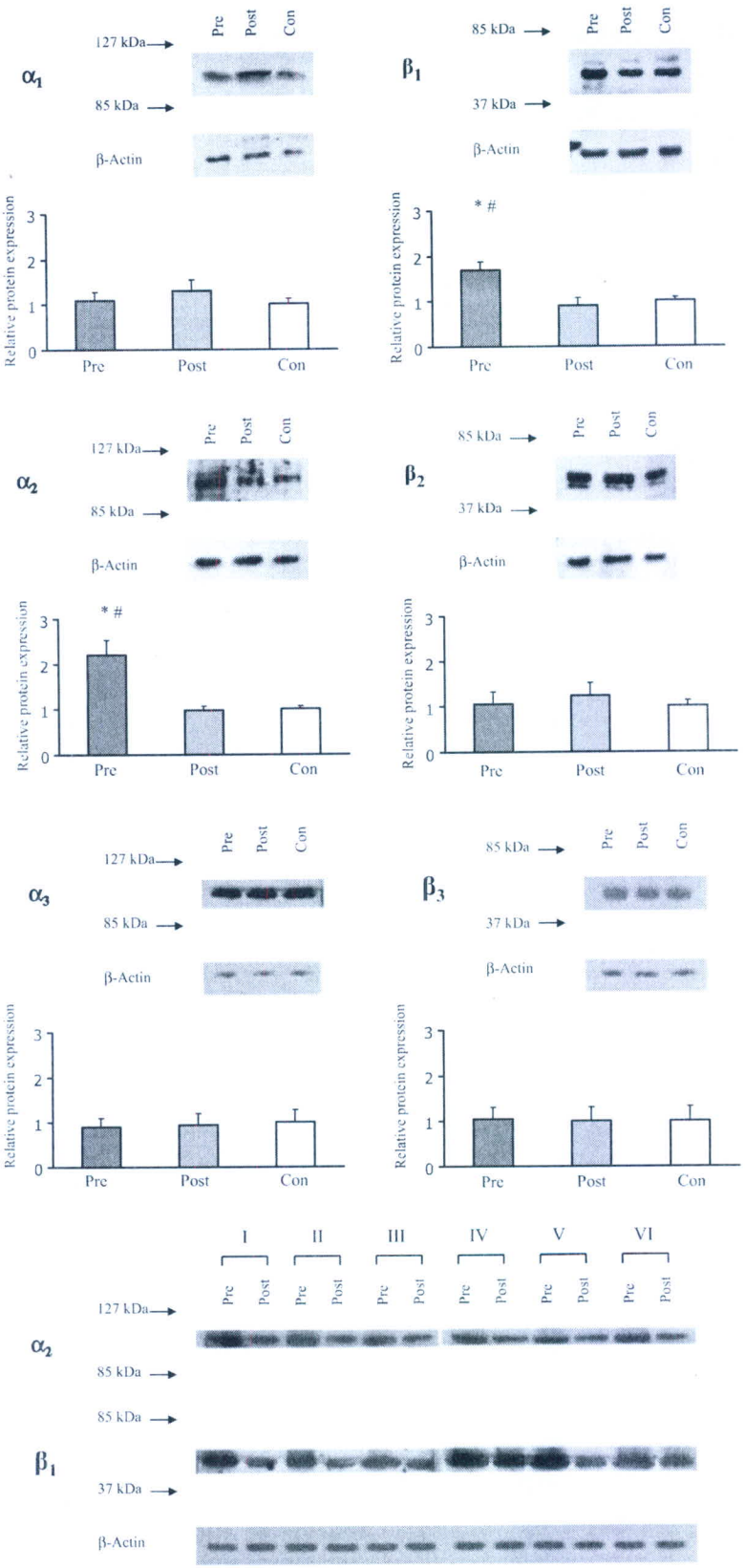

Fig. 4 Immunoblot of skeletal muscle Na^+-K^+-ATPase α_1, α_2, α_3, β_1, β_2 and β_3 isoforms of pre- and postsurgery patients and control subjects. Protein level is expressed relative to that of β actin as mean ± SE (bar). Arrow indicates molecular weight of the protein. The number indicates individual patient. Pre, presurgery $N = 6$; Post, postsurgery $N = 6$; Con, control $N = 10$. $^*P < 0.05$ compared with postsurgery group, $\#P < 0.05$ compared with control group.

study showed that hypokalaemia causes downregulation in the number of Na^+-K^+-ATPase pumps in rat muscle.[25] The relative abundance of Na^+-K^+-ATPase α_2 isoforms in hind limb muscles following 1–4 weeks on a low-K^+ diet showed a progressive decrease and disappeared after 3 weeks.[26] Therefore, the study by Dorup *et al.*[16] showing downregulation of Na^+-K^+-ATPase after aldosterone infusion may not be because of the direct effect of aldosterone, but rather because of hypokalaemia. To clarify this issue, all patients in our study were corrected for hypokalaemia before muscle biopsy. This rules out the effect of hypokalaemia on Na^+-K^+-ATPase activity.

Previous studies in the mouse mammary tumour viral promoter have shown that aldosterone regulates gene expression via its binding to, and activation of, a specific intracellular mineralocorticoid receptor (MR).[27] In the absence of ligand, MR is located primarily in the cytoplasm[28] and associated with chaperone proteins. Upon hormone binding, the MR dissociates from chaperone proteins, undergoes nuclear translocation and interacts with numerous molecular partners in a coordinated and sequential manner to ensure appropriate transcriptional regulation.[29] In kidney cell culture, aldosterone affects Na^+-K^+-ATPase α and β subunits at the transcriptional, translational and transport levels.[15,30] In addition, spironolactone, an aldosterone antagonist, decreases basal α and β subunits of Na^+-K^+-ATPase mRNA levels and hence decreases the relative rate of protein biosynthesis. It also blocks cell response to aldosterone in cultured kidney cells.[30] Ikeda *et al.*[17] have demonstrated that aldosterone directly stimulates Na^+-K^+-ATPase α_1 and β_1 subunit mRNA synthesis and protein accumulation in adult and neonatal rat ventricular cardiocytes grown in defined serum-free media. Several lines of evidence have suggested that MR mediates the action of aldosterone and that it is expressed primarily in epithelial cells such as distal convoluted tubules and collecting tubules.[31,32] In addition, MR expression has been detected in non-epithelial tissues including cardiomyocyte[33,34] and vascular smooth muscle cells.[35] Until now, it was still unclear whether skeletal muscle cells expressed MR. From this point of view, the increase in Na^+ – K^+ ATPase expression observed in this study might possibly be as a result of the direct effect of aldosterone on MR resulting in increased biosynthesis of α_2 and β_1 subunits of Na^+-K^+-ATPase in skeletal muscle cells. Alternatively, this observation might be a secondary response to changes in sodium and/or potassium status in the intracellular compartment mediated by aldosterone.

In patients with Conn's syndrome, total body potassium is likely to be significantly decreased. Unfortunately, because of limitations of study in human, it could not be measured in our study. We are aware that potassium replacement before adrenalectomy may not have completely corrected total body potassium levels, despite the observed normal serum potassium levels. Moreover, the sodium retention associated with hyperaldosteronism, which may not be ameliorated by antihypertensive agents, could increase the intracellular sodium level. Thus, the decreased intracellular potassium and increased intracellular sodium could account in part for the stimulation of Na^+-K^+-ATPase activity in skeletal muscle cells observed in our study. Subsequently, after adrenalectomy, which corrected the high level of aldosterone, the Na^+-K^+-ATPase activity in the skeletal muscle cells was reduced to normal levels. Our observation has a clinical implication for patients with hyperaldosteronism, especially those who cannot undergo surgery, that have increased activity of skeletal muscle Na^+ – K^+ ATPase. They should receive drugs that block the action of aldosterone and/or Na^+ – K^+ ATPase to prevent them from developing hypokalaemia, which partly might be because of potassium uptake into the muscle cells. Equally important, to maintain the equilibrium of total body potassium, adequate potassium intake in spite of normokalaemia should be recommended.

In summary, our study has provided the first evidence that increased plasma aldosterone leads to increased Na^+-K^+-ATPase activity in human skeletal muscles, possibly because of the upregulation of mRNA and protein levels of the α_2 and β_1 (and not α_1, α_3, β_2 and β_3) subunits. Further studies on the detailed mechanisms by which aldosterone activates human skeletal muscle Na^+-K^+-ATPase activity under normal physiological and pathological conditions are necessary to clarify the clinical implications of this hormone.

Acknowledgements

Our grateful appreciation is extended to all patients who participated in this study. We gratefully acknowledge Prof. Michael J. McKenna, Centre for Ageing, Rehabilitation, Exercise and Sport, Victoria University for his invaluable advice on techniques of Na^+-K^+ ATPase measurement in human skeletal muscle cells. We also thank Prof. Prapon Wilairat, Department of Biochemistry, Mahidol University and Mrs. Kristen K. Evans, Department of Physiology, College of Medicine, University of Arizona for their valuable comments and corrections of this manuscript. This work was supported by The Thailand Research Fund and Mahidol University Grant.

Declaration of interest

Nothing to declare.

References

1 Macknight, A.D. & Leaf, A. (1977) Regulation of cellular volume. *Physiological Reviews*, **57**, 510–573.

2 Lingrel, J.B., Van Huysse, J., O'Brien, W. *et al.* (1994) Structure-function studies of the Na,K-ATPase. *Kidney International*, **44**, S32–S39.

3 Vasilets, L.A. & Schwarz, W. (1993) Structure-function relationships of cation binding in the Na+/K(+)-ATPase. *Biochimica et Biophysica Acta*, **1154**, 201–222.

4 Schmalzing, G., Ruhl, K. & Gloor, S.M. (1997) Isoform-specific interactions of Na,K-ATPase subunits are mediated via extracellular domains and carbohydrates. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 1136–1141.

5 Blanco, G. & Mercer, R.W. (1998) Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *The American Journal of Physiology*, **275**, F633–F650.

6 Gick, G.G., Hatala, M.A., Chon, D. *et al.* (1993) Na,K-ATPase in several tissues of the rat: tissue-specific expression of subunit mRNAs and enzyme activity. *The Journal of Membrane Biology*, **131**, 229–236.

7 Hundal, H.S., Maxwell, D.L., Ahmed, A. *et al.* (1994) Subcellular distribution and immunocytochemical localization of Na,K-ATPase subunit isoforms in human skeletal muscle. *Molecular Membrane Biology*, **11**, 255–262.

8 Juel, C., Nielsen, J.J. & Bangsbo, J. (2000) Exercise-induced translocation of Na(+)-K(+) pump subunits to the plasma membrane in human skeletal muscle. *The American Journal of Physiology*, **278**, R1107–R1110.

9 Hundal, H.S., Marette, A., Mitsumoto, Y. *et al.* (1992) Insulin induces translocation of the alpha 2 and beta 1 subunits of the Na+/K(+)-ATPase from intracellular compartments to the plasma membrane in mammalian skeletal muscle. *The Journal of Biological Chemistry*, **267**, 5040–5043.

10 Phakdeekitcharoen, B., Phudhicharoenrat, S., Pookarnjanamorakot, C. *et al.* (2007) Thyroid hormone increases mRNA and protein expression of Na+-K+-ATPase alpha2 and beta1 subunits in human skeletal muscles. *The Journal of Clinical Endocrinology and Metabolism*, **92**, 353–358.

11 Malik, N., Canfield, V.A., Beckers, M.C. *et al.* (1996) Identification of the mammalian Na,K-ATPase 3 subunit. *The Journal of Biological Chemistry*, **271**, 22754–22758.

12 Abriel, H., Hasler, U., Geering, K. *et al.* (1999) Role of the intracellular domain of the beta subunit in Na,K pump function. *Biochimica et Biophysica Acta*, **1418**, 85–96.

13 Petty, K.J., Kokko, J.P. & Marver, D. (1981) Secondary effect of aldosterone on Na-KATPase activity in the rabbit cortical collecting tubule. *The Journal of Clinical Investigation*, **68**, 1514–1521.

14 Binder, H.J., McGlone, F. & Sandle, G.I. (1989) Effects of corticosteroid hormones on the electrophysiology of rat distal colon: implications for Na+ and K+ transport. *The Journal of Physiology*, **410**, 425–441.

15 Verrey, F., Kraehenbuhl, J.P. & Rossier, B.C. (1989) Aldosterone induces a rapid increase in the rate of Na,K-ATPase gene transcription in cultured kidney cells. *Molecular Endocrinology*, **3**, 1369–1376.

16 Dorup, I. & Clausen, T. (1997) Effects of adrenal steroids on the concentration of Na(+)-K+ pumps in rat skeletal muscle. *The Journal of Endocrinology*, **152**, 49–57.

17 Ikeda, U., Hyman, R., Smith, T.W. *et al.* (1991) Aldosterone-mediated regulation of Na+, K(+)-ATPase gene expression in adult and neonatal rat cardiocytes. *The Journal of Biological Chemistry*, **266**, 12058–12066.

18 Norgaard, A., Kjeldsen, K. & Hansen, O. (1984) (Na+ + K+)-ATPase activity of crude homogenates of rat skeletal muscle as estimated from their K+-dependent 3-O-methylfluorescein phosphatase activity. *Biochimica et Biophysica Acta*, **770**, 203–209.

19 Nordsborg, N., Bangsbo, J. & Pilegaard, H. (2003) Effect of high-intensity training on exercise-induced gene expression specific to ion homeostasis and metabolism. *The Journal of Apply Physiology*, **95**, 1201–1206.

20 Gennari, F.J. (1998) Hypokalaemia. *New England Journal of Medicine*, **339**, 451–458.

21 Turnamian, S.G. & Binder, H.J. (1989) Regulation of active sodium and potassium transport in the distal colon of the rat. Role of the aldosterone and glucocorticoid receptors. *The Journal of Clinical Investigation*, **84**, 1924–1929.

22 Wehling, M. & Theisen, K. (1990) Extrarenal receptor-effector-mechanisms for aldosterone: the sequence of effects on the cellular electrolyte transport in human lymphocytes and their implications for disorders of the water and electrolyte balances. *Acta Endocrinologica*, **123**, 385–394.

23 Liu, X. & Songu-Mize, E. (1997) Alterations in alpha subunit expression of cardiac Na+,K+-ATPase in spontaneously hypertensive rats: effect of antihypertensive therapy. *European Journal of Pharmacology*, **327**, 151–156.

24 Pan, M. & Janis, R.A. (1984) Stimulation of Na+,K+-ATPase of isolated smooth muscle membranes by the Ca2 + channel inhibitors, nimodipine and nitrendipine. *Biochemical Pharmacology*, **33**, 787–791.

25 Clausen, T., Hansen, O., Kjeldsen, K. *et al.* (1982) Effect of age, potassium depletion and denervation on specific displaceable [3H]ouabain binding in rat skeletal muscle in vivo. *The Journal of Physiology*, **333**, 367–381.

26 Hsu, Y.M. & Guidotti, G. (1991) Effects of hypokalaemia on the properties and expression of the (Na+,K+)-ATPase of rat skeletal muscle. *The Journal of Biological Chemistry*, **266**, 427–433.

27 Arriza, J.L., Weinberger, C., Cerelli, G. *et al.* (1987) Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science*, **237**, 268–275.

28 Lombes, M., Binart, N., Delahaye, F. *et al.* (1994) Differential intracellular localization of human mineralocorticosteroid receptor on binding of agonists and antagonists. *The Biochemical Journal*, **302** (Pt 1), 191–197.

29 Viengchareun, S., Le Menuet, D., Martinerie, L. *et al.* (2007) The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. *Nuclear Receptor Signaling*, **5**, e012.

30 Verrey, F., Schaerer, E., Zoerkler, P. *et al.* (1987) Regulation by aldosterone of Na+,K+-ATPase mRNAs, protein synthesis, and sodium transport in cultured kidney cells. *The Journal of Cell Biology*, **104**, 1231–1237.

31 Krozowski, Z.S., Rundle, S.E., Wallace, C. *et al.* (1989) Immunolocalization of renal mineralocorticoid receptors with an antiserum against a peptide deduced from the complementary deoxyribonucleic acid sequence. *Endocrinology*, **125**, 192–198.

32 Lombes, M., Farman, N., Oblin, M.E. *et al.* (1990) Immunohistochemical localization of renal mineralocorticoid receptor by using an anti-idiotypic antibody that is an internal image of aldosterone. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 1086–1088.

33 Barnett, C.A. & Pritchett, E.L. (1988) Detection of corticosteroid type I binding sites in heart. *Molecular and Cellular Endocrinology*, **56**, 191–198.

34 Lombes, M., Oblin, M.E., Gasc, J.M. *et al.* (1992) Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. *Circulation Research*, **71**, 503–510.

35 Nakamura, Y., Suzuki, S., Suzuki, T. *et al.* (2006) MDM2: a novel mineralocorticoid-responsive gene involved in aldosterone-induced human vascular structural remodeling. *The American Journal of Pathology*, **169**, 362–371.

Urine potassium per hour as a marker for renal potassium losses

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Abstract

Background: Hypokalemia, serum potassium (K) < 3.5 mEq/L, is a serious and common clinical problem. The traditional diagnosis of renal potassium losses is 24-hr urine potassium ($24U_K$) $\geq$ 20 mEq/day during hypokalemia. Immediate replacement of potassium is often required to prevent complication but may normalize serum K during 24-hr urine collection and render the test inconclusive.

Patients and Method: The authors examined the ability of urinary indices including $24U_K$, transtubular potassium gradient (TTKG), fractional excretion of potassium (FE_K), urine potassium-creatinine ratio ($U_{K/Cr}$) and spot U_K and introduced urine potassium per hour during the first 8 hours (U_K/hr) as a novel index for evaluation of hypokalemia during treatment. Any serum K level $\geq$ 4 mEq/L during urine collection was defined as normalized serum K. In the present study, the final classification of renal K losses in non-normalized 24-hr serum K group was made when $24U_K \geq 20$ mEq/day. In normalized group, the final classification of renal or non-renal K losses was based on the majority of the results of 4 urine indices including TTKG, FE_K, $U_{K/Cr}$, and spot U_K.

Results: Of 61 patients (renal:non-renal = 50:11), 51% and 18% met the criteria of normalized 24-hr and 8-hr serum K. Over all, the U_K/hr $\geq$ 0.9 mEq/hr can indicate renal K losses with a sensitivity of 96% and specificity of 72.7% compared with the $24U_K \geq 20$ mEq/day of 100% and 54.5%, respectively. In a subgroup of normalized 24-hr serum K, the sensitivity and specificity of U_K/hr = 95.5% and 77.8% whereas $24U_K$ = 100% and 44.4%, respectively.

Conclusion: U_K/hr is a new practical, simple, and reliable marker that can be applied to evaluate hypokalemic patients during treatment with comparable sensitivity and specificity with $24U_K$.

Keywords: Hypokalemia, urine potassium per hour (U_K/hr), 24-hour urine potassium ($24U_K$), transtubular potassium gradient (TTKG), fractional excretion of potassium (FE_K), urine potassium-creatinine ratio ($U_{K/Cr}$)

Introduction

Hypokalemia is a commonly encountered clinical problem[1]. Immediate treatment is sometimes necessary to avoid a life threatening condition[2]. The etiology of hypokalemia is often not immediately obvious at initial presentation. Most hypokalemia results from potassium depletion induced by abnormal losses of potassium either due to renal or non-renal losses[3-7]. The kidney plays a major role in long-term potassium homeostasis[8-10]. The measurement of urinary potassium excretion is very helpful in differentiation of renal from non-renal K losses and assignment of appropriate treatment. 24-hour urine potassium ($24U_K$) collection is often used as the gold standard[9,11]. Urinary K (U_K) excretion of more than 20 mEq/24-hr during hypokalemia is indicative of excessive renal K losses[12,13]. In contrast, non-renal K wasting is suspected when urinary K excretion is less than 20 mEq/24-hr[11,14]. However, this is not feasible in many cases. It is sometimes difficult to obtain complete 24 hr urine collection. Moreover, the serum K levels during 24-hour urine collection might not be persistently low due to treatment. Excess of potassium from treatment would result in excretion of K into urine. This may affect the total excretion of urinary potassium and makes the interpretation of 24-hour urine K results uncertain[12,15].

Many authors tried to employ other noninvasive indices such as transtubular potassium concentration gradient (TTKG)[16], fractional excretion of potassium (FE_K)[17], urine potassium-creatinine ratio ($U_{K/Cr}$)[18] and spot U_K[13] to determine the diagnosis. These parameters are simpler but may be less accurate compared to a 24-hour urine collection, since these parameters assess potassium excretion only at a single point in time. No study has been formally evaluated the accuracy of these markers in a large group of patients. Hence, the authors conducted a study to validate the previously known

urine indices and introduced a new urine index that is both simple and reliable in indicating renal potassium losses in hypokalemic patients during treatment. In the present study, the authors evaluated the urine potassium per hour during the first 8 hours (U_K/hr) and compared this value with $24U_K$ and other urinary indices.

Subjects and Method

Subjects

All untreated hypokalemic patients were recruited from the emergency room, medicine and surgery departments. Initially, the etiology of hypokalemia was unknown. All enrolled patients followed the protocol of the present study. Hypokalemia was defined as a serum K level below 3.5 mEq/L. The exclusion criteria were serum creatinine > 1.2 mg/dL, previous renal disease, patients with shock, congestive heart failure, nephrotic syndrome, or cirrhosis. All participants gave their written informed consent after receiving oral and written information concerning the present study according to the Declaration of Helsinki II. The study protocol was approved by the Ethical Committee for Research Involving Human Subjects of the Ramathibodi Hospital, Mahidol University (ID 08-48-16).

The final diagnosis of the cause of hypokalemia in each patient was assigned after the clinical course and careful investigations. The definite diagnoses of various diseases causing hypokalemia were as follows. Hypomagnesemia was defined by serum magnesium < 1.4 mEq/L on two occasions. Solute diuresis was defined by (1) the urine osmolality above 300 mOsm/kg, and (2) total solute excretion above 900 mOsm/day. The diagnosis of diabetic ketoacidosis was based on (1) blood glucose ≥ 250 mg/dL, (2) ketosis, and (3) metabolic acidosis (arterial pH < 7.3). The diagnosis of a hyperglycemic hyperosmolar state was based on (1) blood glucose ≥ 600 mg/dL, (2) hyperosmolality (serum osmolality > 350 mOsm/kg), and (3) absence of or mild acidosis and ketonemia. The diagnosis of hyperaldosteronism was a plasma aldosterone level > 160 pg/mL (normal 10-160 pg/mL in a supine position) on two occasions. Thyrotoxic periodic

paralysis was defined by (1) hyperthyroidism [(i) serum TSH < 0.3 μIU/mL (normal 0.3-4.0), and (ii) free T4 > 2.0 ng/dL (normal 0.9-2.0), or T3 > 160 ng/dL (normal 80-160), or total T4 > 12 μg/dL (normal 4.0-12.0)], (2) attacks occurring suddenly with generalized weakness and preserved consciousness, and (3) episodes association with hypokalemia. The diagnosis of distal renal tubular acidosis was based on (1) hyperchloremia metabolic acidosis with normal GFR (> 60 mL/min), and (2) abnormal long acid loading test which was defined as failure to acidify urine (pH > 5.5) and urine excretion of ammonia < 50 mEq/day in the presence of systemic acidosis (blood pH < 7.35) [11].

Method

Patient characteristics were documented. Blood samples were initially collected from patients for determination of electrolytes, blood urea nitrogen, creatinine, calcium, phosphate, magnesium, albumin, uric acid and osmolality. Serum K was measured every 8 hours. The first voiding urine was collected for measuring TTKG, FE_K, $U_{K/Cr}$, and spot U_K. Then, 24 hour urine was collected every 8 hour interval for volume, potassium, sodium, creatinine, calcium, phosphate, and magnesium measurements.

After the first voiding urine collection, the potassium supplements were given. The treatment protocol for mild hypokalemia (K 3.0 to < 3.5 mEq/L) was oral potassium chloride (KCl) elixir 20 mEq every 8 hours. In moderate hypokalemia (K 2.5 to < 3.0 mEq/L), oral KCl elixir 40 mEq every 8 hours plus intravenous KCl infusion at the rate 5 mEq/hr were given. In severe hypokalemia (K < 2.5 mEq/L) or symptomatic hypokalemia (e.g. arrhythmias), oral KCl elixir 40 mEq every 8 hours plus intravenous KCl infusion at the rate 10 mEq/hr were given with electrocardiographic monitoring. Nevertheless, the

judgment of the potassium supplement rate was based on the clinician's decision against clinical setting and the risk of hyperkalemia.

The U_K/hr during the first 8 hours was calculated by using the concentration of urine potassium during the first 8 hours (mEq/L) multiplied by urine volume during the first 8 hours (L) and divided by 8. TTKG, FE_K and $U_{K/Cr}$ were calculated from the equations [(urine/serum K)/(urine/serum osmolality)][16], [(urine/serum K)/(urine/serum creatinine)][17], and (urine K/urine creatinine)[18], respectively. Patients who had any serum K levels $\geq$ 4 mEq/L during urine collection were considered as a normalized serum K group, otherwise they were considered as a non-normalized serum K group. The cutoff values to indicate renal or non-renal K losses of $24U_K$, TTKG, FE_K, $U_{K/Cr}$ and spot U_K were 20 mEq/day[12], 2.5[16], 6.5%[17], 2.5 mmol/mmol[18] and 20 mEq/L[13], respectively.

In the present study, the final classification of renal or non-renal K losses in each patient was as follows. In non-normalized 24-hr serum K group (serum K persistently < 4 mEq/L throughout 24 hours), the final classification of renal K losses was made when $24U_K \geq 20$ mEq/day. In normalized 24-hr serum K group, the final classification of renal or non-renal K losses was based on the majority of the results of 4 urine indices including TTKG, FE_K, $U_{K/Cr}$, and spot U_K. In case that the final classification of the patient was equivocal (renal:non-renal = 2:2), the patient would be classified as a non-conclusive group. The final classification of each patient was correlated with the authors' clinical diagnosis.

Statistical analysis

The mean ± standard deviation (SD) was used to describe the baseline characteristics and the mean ± standard error of mean (SEM) was calculated for patient diagnostic measures. The statistical significance of the difference in these characteristics across those with renal and non-renal potassium losses was examined by using Mann Whitney U- test or *t* test (continuous variable and depend on the data distribution) and chi-square or Fishers' exact test (categorical variable). The cut off point of all urine indices were calculated from the receiver-operating characteristic (ROC) curve and the coordination table of the curve for the value of the highest sensitivity and specificity. The difference was considered significant if the *P*-value was < 0.05.

Results

Seventy-five patients with hypokalemia were included in the present study. Sixty-one patients completed the present study. There were 19 males (31.2%) and 42 females (68.9%). The baseline characteristics are summarized in **Table 1**. Of 61 patients, 50 (82%) were classified as renal K losses and 11 (18%) as non-renal K losses. No patient was classified as a non-conclusive group in the present study. There were no significant differences in the mean serum K level at presentation, age, and serum creatinine concentration between the two groups ($P > 0.05$). Of 61 patients, only 30 (49.2%) met the criteria for non-normalized 24-hr serum K. In contrast, 50 (82 %) patients met the criteria for non-normalized 8-hr serum K **(Table 1)**. The number of patients between renal and non-renal potassium losses in the non-normalized 24-hr serum K group was the factor that was significantly different ($P = 0.02$). Total 24-hr K supplement in renal K loss group was higher than that of non-renal K loss group ($P = 0.01$).

In the present study, drug induced renal potassium wasting (e.g. amphotericin B, aminoglycoside, ifosphamide, etc.) was the leading cause of renal K losses **(Table 2)**. For non-renal K losses, the major cause of disease was diarrhea. 2 of 11 (18.2%) patients in the non-renal K loss group were due to cellular potassium shift (thyrotoxic periodic palalysis).

TTKG can only be interpreted correctly when urine osmolality is higher than serum osmolality[16]. In this case, TTKG could be interpreted in 52 out of 61 patients. As expected in every urine index, the urine excretion of potassium in the renal K loss group was higher than that of the non-renal K loss group ($P < 0.05$) **(Table 3)**.

Scatter plots were used for data visualization to explore the cutoff values for each diagnostic measure compared with final classification **(Figure 1A)**. Of 61 patients, fifty-five (90.2%) had $24U_K \geq 20$ mEq/day. The other six (9.8%) had $24U_K < 20$mEq/day **(Figure 1A)**. In the group in which $24U_K \geq 20$ mEq/day, 50 out of 55 (90.9%) patients had a final classification of hypokalemia from renal K losses. The other five (9.1%) were classified as non-renal K losses. All of the latter were in the normalized 24-hr serum K group. The sensitivity and specificity of 24 $U_K \geq 20$ mEq/day to indicate renal K losses were 100% and 54.5%, respectively **(Table 3)**.

The best diagnostic cutoff value for U_K/hr in the present study was 0.9 mEq/hr, from the optimal point of the ROC curve **(Figure 2B)**. Of 61 patients, 51 (83.6 %) had $U_K/hr \geq 0.9$ mEq/hr. The other 10 (16.4%) had $U_K/hr < 0.9$ mEq/hr **(Figure 1B)**. In the group in which $U_K/hr \geq 0.9$ mEq/hr, 48 out of 51 (94.1%) patients had a final classification of renal K losses. The other three (5.9%) were classified as non-renal K losses. All of the latter were in the normalized 8-hr serum K group. The sensitivity and specificity of this new index was 96 % and 72.7 %, respectively. A comparison of the areas under the ROC curve between $24U_K$ and U_K/hr are shown in **Figure 2**. U_K/hr tended to have higher AUC than $24U_K$ (0.86 vs. 0.84). In the subgroup of patients with normalized 24-hr serum K, the authors identified the reduction of $24U_K$ specificity from total to subgroup, 54.5% to 44.4 %, with the increase of U_K/hr specificity from 72.7 to 77.8 % **(Table 3 and 4)**.

In another view, of hypokalemic patients who had a final classification of non-renal K losses, only 6 out of 11 (54.5%) patients had $24U_K < 20$ mEq/day whereas 8 out of 11 (72.7%) had $U_K/hr < 0.9$ mEq/hr. Two patients diagnosed as cellular K shift (thyrotoxic periodic paralysis) were misdiagnosed by using $24U_K$ and U_K/hr. Moreover,

TTKG and spot U_K were unreliable for diagnosis of cellular K shift. Only $U_{K/Cr}$ and FE_K could be used to identify these two patients correctly.

For other indices, TTKG, FE_K, $U_{K/Cr}$, and spot U_K were evaluated for their accuracy by using the previously mentioned cutoff point referred to in many studies **(Table 3)**.

To identify the best cutoff value for each urine index in the present study, the optimal point of the ROC curves were plotted. The best cutoff values for 24 U_K, TTKG, FE_K, $U_{K/Cr}$, and spot U_K were 23 mEq/day, 2.7, 5.5%, 3.0 mmol/mmol, and 10.5 mEq/L, respectively. With the new cutoff values, the new sensitivity and specificity of each urine index were 94%, 93%, 95.9%, 85.7% and 90%, respectively, and 63.6%, 66.7%, 63.6%, 72.7% and 45.6%, respectively **(data not shown)**.

Discussion

Hypokalemia is a common clinical problem arising from diverse etiologies[19]. Even mild or moderate hypokalemia increases the risks of morbidity and mortality in patients especially with cardiovascular diseases[2]. As a result, when hypokalemia is identified, the underlying cause should be sought and treated. The traditional approach to distinguish between renal and non-renal causes of hypokalemia is based on a 24-hour urine potassium[9,12,14]. In the setting of hypokalemia, a urine potassium that is more than 20 mEq/day suggests that there is renal cause of potassium wasting[9,12,14]. Levels below these values indicate that there are non-renal causes for potassium depletion. However, obtaining the 24 hr urine potassium is sometimes difficult in clinical practice because it takes too long for urine collection. Most important, therapy with potassium must be given promptly in most situations[15,20]. Hence, serum potassium during 24-hour urine collection might not be persistently low because of potassium supplementation. This situation makes the interpretation unclear. To avoid this limitation, the authors proposed a new index which assessed urine potassium for a period of time, not only one time point like TTKG[16], FE_K[17], $U_{K/Cr}$[18], or spot U_K[13], and more practical than the 24 hour urine collection[12]. In the present study, the authors evaluated the roles of U_K/hr in the first 8 hours to discriminate renal from non-renal K losses compared with other urine indices.

Ideally, a 24-hr urine potassium collection should be performed without any potassium supplement. In the reality, this strategy may not be safe for the patients since the complications of hypokalemia are sometimes severe and sometimes life threatening[2]. Thus, evaluation of hypokalemia during treatment is essential in clinical practice. From the data shown in **Table 3**, 24 U_K and U_K/hr were the markers that had high sensitivity and

specificity in predicting renal potassium losses in the present study. Regarding their accuracies in predicting renal K losses, U_K/hr > 0.9 mEq/hr can indicate renal K losses with a sensitivity of 96 % and a specificity of 72.7 % whereas $24U_K \geq 20$ mEq/day had a sensitivity and specificity of 100% and 54.5 %, respectively. A low specificity of $24U_K$ may result from serum K levels that were not persistently low during the 24-hr urine collection. Only 30 out of 61 (49.2%) of 24-hr urine collections met the criteria for non-normalized serum K (serum K persistently < 4 mEq/L throughout 24 hours) whereas 50 out of 61 (82%) of 8-hr urine collection met the criteria for non-normalized serum K (serum K persistently < 4 mEq/L throughout the first 8 hours). This data indicated the chance that the authors would collect a complete $24U_K$ during a persistently low serum potassium period and the chance that the authors could reliably interpret these data was generally less than a half. In contrast with $24U_K$, the shorter duration in urine collection of U_K/hr resulted in a greater number of patients who met the criteria for non-normalized serum K and made the data more reliable for interpretation. Interestingly, U_K/hr is a new index that is much more practical, convenient and takes less time than the conventional 24-hr urine collection. This marker not only had comparable sensitivity, but also had a higher specificity than $24U_K$ especially in cases that serum K was not persistently low throughout the 24-hr urine collection.

Based on Ethier J et al.[16], TTKG is a simple index to evaluate renal K losses. However, it still has some limitations. One must rely on another index (osmolality) to calculate the TTKG. Moreover, TTKG is invalid if the urine osmolality is lower than the plasma osmolality. In the present study, the authors could use only 85% of TTKG values because 9 patients had the urine osmolality lower than the plasma osmolality. When

compared with U_K/hr, the number of patients that can be interpreted by U_K/hr was more than those by TTKG and U_K/hr was more reliable with a higher specificity in predicting renal K losses.

For FE_K, $U_{K/Cr}$, and spot urine K, although these noninvasive parameters are simple to use, they are less accurate compared with $24U_K$ or U_K/hr, because the former parameters assess potassium excretion only at one time point. In the present study, although the authors used the best cutoff value to determine the best sensitivity and best specificity of each marker, FE_K, $U_{K/Cr}$, and spot U_K still had low sensitivities, specificities and area under the curve of ROC when compared with U_K/hr.

U_K/hr had a limitation in diagnosis of patients who had hypokalemia from thyrotoxic periodic paralysis. From the authors' previous study[21], it was found that 9 out of 11 of our thyrotoxic periodic paralysis patients recovered from hypokalemia within 24 hours. 6 out of 11 patients recovered from hypokalemia within 8 hours. The authors speculated that both a rapid change of transcellular potassium from hypokalemia to normokalemia or even hyperkalemia and potassium supplementation from treatment might increase the urine potassium excretion and, thus, made the test inaccurate. In TPP patients, the authors suggest that the clinical setting and awareness of the disease are important for the definite diagnosis.

In conclusion, the present study demonstrated that U_K/hr is a new practical, simple, and reliable marker that can be applied to evaluate renal K losses in hypokalemic patients during treatment with comparable sensitivity and specificity with $24U_K$.

Acknowledgements

The authors wish to thank all patients who participated in our studies and to many physicians who supplied specimens and clinical information on their patients. The suggestions and reviews of this manuscript by Dr. Chagriya Kitiyakara, Division of Nephrology and the statistical analysis by Mrs. Umaporn Udomsubpayakul, Clinical Epidemiology Unit, Ramathibodi Hospital are greatly appreciated. This project was supported by The Thailand Research Fund and The Ramathibodi Research Fund, Mahidol University, Bangkok, Thailand.

References

1 Gennari FJ. Hypokalemia. N Engl J Med 1998; 339: 451-8.

2 Schulman M, Narins RG. Hypokalemia and cardiovascular disease. Am J Cardiol 1990; 65: 4E-9E; discussion 22E-3E.

3 McCleave DJ, Phillips PJ, Vedig AE. Compartmental shift of potassium--a result of sympathomimetic overdose. Aust N Z J Med 1978; 8: 180-3.

4 Jamison RL, Ross JC, Kempson RL, Sufit CR, Parker TE. Surreptitious diuretic ingestion and pseudo-Bartter's syndrome. Am J Med 1982; 73: 142-7.

5 Adrogue HJ, Madias NE. Changes in plasma potassium concentration during acute acid-base disturbances. Am J Med 1981; 71: 456-67.

6 Ober KP. Thyrotoxic periodic paralysis in the United States. Report of 7 cases and review of the literature. Medicine (Baltimore) 1992; 71: 109-20.

7 Jacob H. Potassium deficiency occurring during treatment of diabetic acidosis. JAMA 1946; 131: 1186-9.

8 Squires RD, Huth EJ. Experimental potassium depletion in normal human subjects. I. Relation of ionic intakes to the renal conservation of potassium. J Clin Invest 1959; 38: 1134-48.

9 Mount D, Zandi-Nejad K. Disorder of potassium balance. In: Brenner BM, Rector FC, (eds). Brenner & Rector's the kidney. 7th ed. Philadelphia: Saunders; 2004: 997-1040.

10 Field MJ, Giebisch GJ. Hormonal control of renal potassium excretion. Kidney Int 1985; 27: 379-87.

11 Rose BD, Post TW. Introduction to disorder of potassium balance. In: Rose BD, (ed). Clinical physiology of acid-base and electrolyte disorders. 5th ed. New York: McGraw-Hill; 2001: 289-94.

12 Narins RG, Jones ER, Stom MC, Rudnick MR, Bastl CP. Diagnostic strategies in disorders of fluid, electrolyte and acid-base homeostasis. Am J Med 1982; 72: 496-520.

13 Halperin ML, Kamel KS. Potassium. Lancet 1998; 352: 135-40.

14 Kone BC. Hypokalemia. In: DuBose TD, Hamm LL, (eds). Acid-base and electrolyte disorders : a companion to Brenner & Rector's The kidney. Philadelphia, Pa.: Saunders; 2002: 381-94.

15 Griggs RC, Resnick J, Engel WK. Intravenous treatment of hypokalemic periodic paralysis. Arch Neurol 1983; 40: 539-40.

16 Ethier JH, Kamel KS, Magner PO, Lemann J, Jr., Halperin ML. The transtubular potassium concentration in patients with hypokalemia and hyperkalemia. Am J Kidney Dis 1990; 15: 309-15.

17 Elisaf M, Siamopoulos KC. Fractional excretion of potassium in normal subjects and in patients with hypokalaemia. Postgrad Med J 1995; 71: 211-2.

18 Lin SH, Lin YF, Chen DT, Chu P, Hsu CW, Halperin ML. Laboratory tests to determine the cause of hypokalemia and paralysis. Arch Intern Med 2004; 164: 1561-6.

19 Gennari FJ. Disorders of potassium metabolism. In: Suki WN, Massry SG, (eds). Suki and Massry's Therapy of renal diseases and related disorders. 3rd ed. ed. Dordrecht ; London: Kluwer Academic; 1997: 53-84.

20 Rimmer JM, Horn JF, Gennari FJ. Hyperkalemia as a complication of drug therapy. Arch Intern Med 1987; 147: 867-9.

21 Phakdeekitcharoen B, Ruangraksa C, Radinahamed P. Hypokalaemia and paralysis in the Thai population. Nephrol Dial Transplant 2004; 19: 2013-8.

Figure legends

Figure 1. Demonstrates the urine indices and baseline serum potassium level in all patients. **(A)** 24 hour urine potassium ($24U_K$), **(B)** urine potassium per hour (U_K/hr), **(C)** Transtubular potassium concentration gradient (TTKG), **(D)** Fractional excretion of potassium (FE_K), **(E)** Urine potassium-creatinine ratio ($U_{K/Cr}$) and **(F)** Spot urine potassium (spot U_K). The dash line indicates the cutoff value (from reference studies) to discriminate renal from non-renal potassuim losses for each urine index.

● = Renal K loss

○ = Non-renal K loss

Figure 2. Demonstrates receiver-operating characteristic (ROC) curve and area under the curve.

(A) 24 hour urine potassium ($24U_K$), **(B)** urine potassium per hour (U_K/hr). The dash line indicates the optimal cutoff value to discriminate renal from non-renal potassium losses, (cutoff value = 0.9 mmol/hr).

Table 1: Baseline characteristics of the patients

	Total	Mean ± SD		
		Renal	Non-renal	*P* value
Number	61 (100%)	50 (82%)	11 (18%)	
Age (mean ± SD, year)	50.9 ± 16.7	52.0 ± 16.4	45.8 ± 18.0	NS
Sex (F:M)	42:19	34:16	8:3	NS
Serum K at presentation (mean ± SD, mEq/L)	2.94 ± 0.43	2.88 ± 0.41	3.20 ± 0.22	NS
Serum creatinine (mean ± SD, mg/dL)	0.8 ± 0.3	0.8 ± 0.3	0.9 ± 0.2	NS
Number of patients in non-normalized 24-hr serum K †	30/61 (49.2%)	28	2	0.02
Number of patients in non-normalized 8-hr serum K ††	50/61 (82%)	41	9	NS
Total 24-hr K supplement (mean ± SD, mEq/day)	94 ± 85	104 ± 89	49 ± 47	0.01

† non-normalized 24-hr serum K = patients who had serum K level < 4 mmol/L throughout 24 hours

†† non-normalized 8-hr serum K = patients who had serum K level < 4 mmol/L throughout the first 8 hours

NS = Not significant

Table 2: The definite causes of hypokalemia in all patients

Renal losses		**50 cases**
	Hypomagnesemia	8
	Diuretics	9
	Drug induced	12
	Distal renal tubular acidosis	2
	Solute diuresis/DKA*/Hyperosmolar	9
	Vomiting	3
	Hyperaldosteronism	7
Non-renal losses		**11 cases**
	Diarrhea	5
	Low intake	4
	Thyrotoxicosis periodic paralysis	2
Total		**61 cases**

*DKA = diabetic ketoacidosis

Table 3: Sensitivities and specificities of various urine indices in predicting renal K losses in all patients

Index	Diagnosis (Mean ± SEM)		*P* value	Cutoff value	Reference	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Renal (R)	Non-renal (NR)							
$24U_K$ (mEq/day) R:NR = 50:11	54.0 ± 4.0	26.6 ± 7.0	0.003	20	Narins et al. 1982	100	54.5	90.9	100
U_K/hr (mEq/hr) R:NR = 50:11	3.5 ± 0.3	1.3 ± 0.6	0.007	0.9	This study	96	72.7	94.1	80
TTKG *R:NR = 43:9	6.8 ± 0.8	2.7 ± 0.5	0.001	2.5	Ethier et al. 1990	95.3	44.4	89.1	66.7
FE_K (%) **R:NR = 49:11	22.4 ± 2.5	11.4 ± 4.7	0.03	6.5	Elisaf and Siamopoulos 1995	93.9	63.6	92	70
$U_{K/Cr}$ (mmol/mmol) **R:NR = 49:11	9.1 ± 1.4	3.4 ± 1.1	0.02	2.5	Lin et al. 2004	85.7	54.5	89.4	46.2
Spot U_K (mEq/L) R:NR = 50:11	27.5 ± 2.3	13.2 ± 2.3	0.001	20	Halperin and Kamel 1998	62	81.8	93.9	32.1

$24U_K$ = 24 hour urine potassium, U_K/hr = urine potassium per hour, TTKG = transtubular potassium concentration gradient, FE_K = fractional excretion of potassium, $U_{K/Cr}$ = urine potassium-creatinine ratio, Spot U_K = spot urine potassium, PPV= positive predictive value, NPV= negative predictive value, SEM = standard error of mean, R = renal, NR = non-renal *Interpreted only when urine osmolality is higher than serum osmolality **Missing one value of serum creatinine

Table 4: Sensitivities and specificities of $24U_K$ and U_K/hr in predicting renal K losses in subgroup of normalized 24-hr serum K patients.

Index	Diagnosis (Mean ± SEM)		*P* value	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Renal N = 22	Non-renal N = 9						
$24U_K$ (mEq/day)	53.7 ± 7.4	28.9 ± 8.5	0.039	20	100	44.4	81.5	100
U_K/hr (mEq/hr)	3.3 ± 0.4	1.5 ± 0.8	0.049	0.9	95.5	77.8	91.3	87.5

$24U_K$ = 24 hour urine potassium, U_K/hr = urine potassium per hour

PPV= positive predictive value, NPV= negative predictive value, SEM = standard error of mean

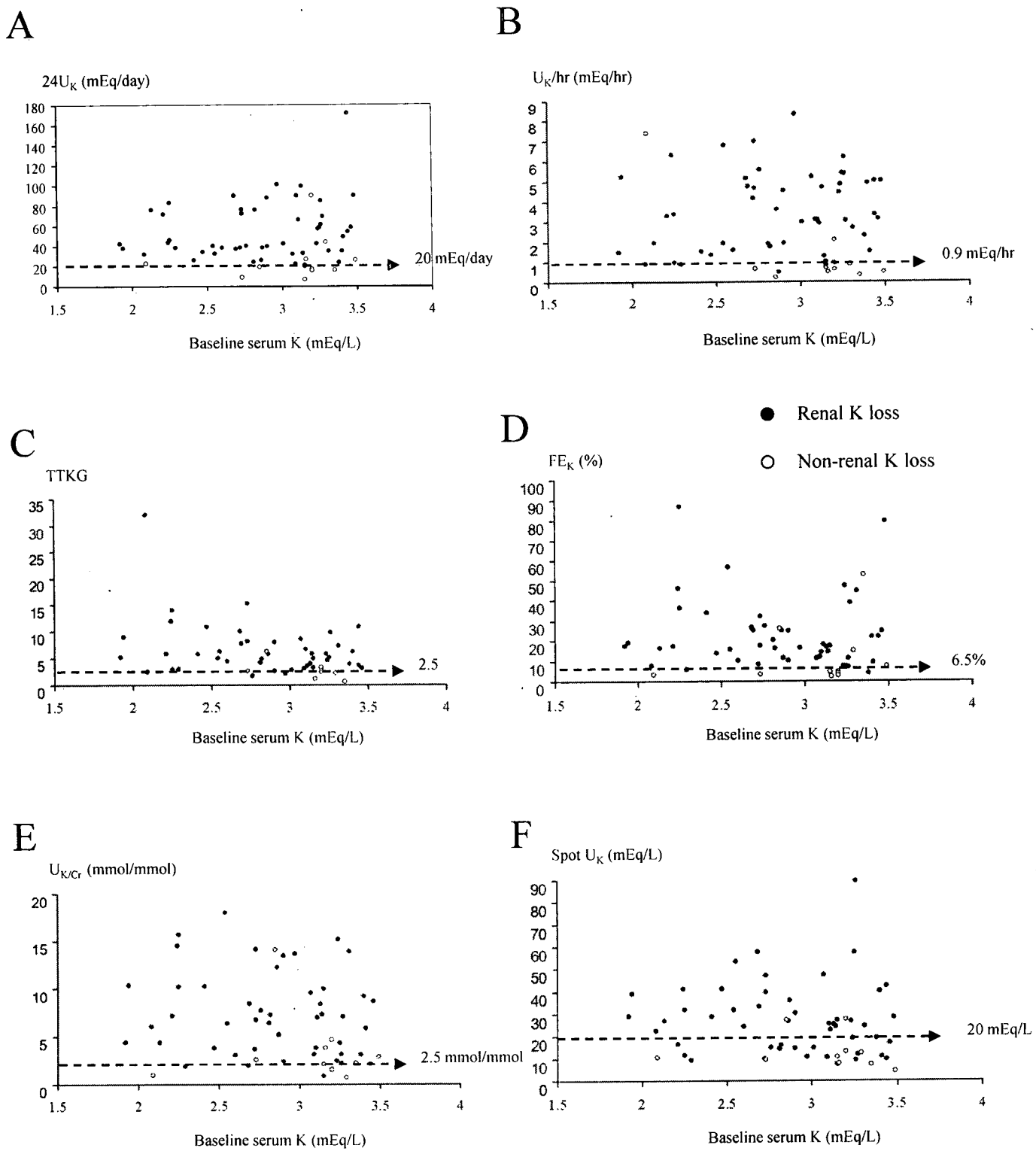

Figure 1: The urine indices and baseline serum potassium level in all patients

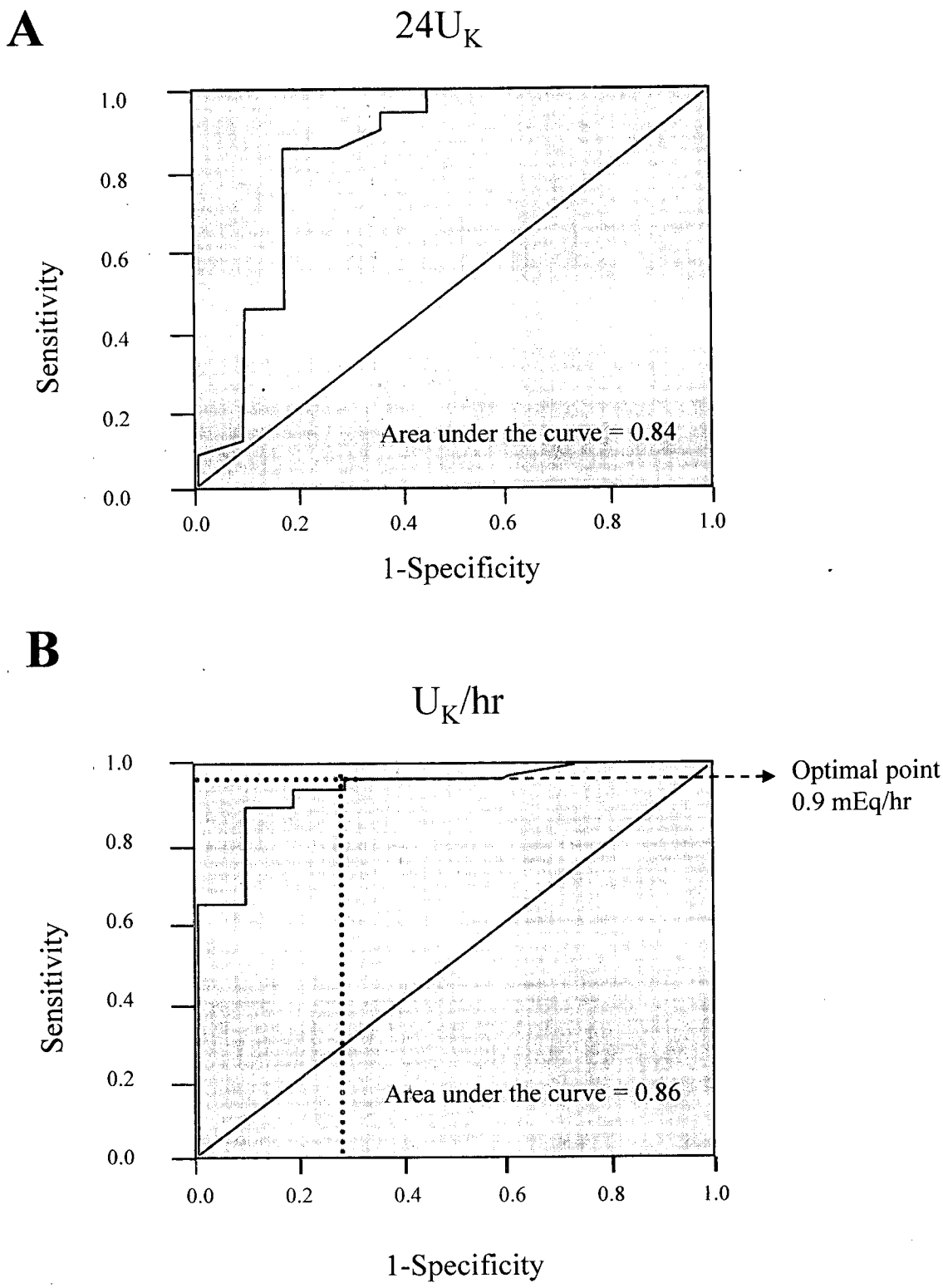

Figure 2: ROC curve and area under the curve of $24U_K$ (A) and U_K/hr (B)