Abnormal Kinetics of Erythrocyte Sodium Lithium Countertransport in Patients with Diabetic Nephropathy in Thailand

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Background: *In essential hypertension and diabetic nephropathy, sodium-lithium counter transport (Na/Li CT) is an inherited marker for metabolic influences of cardiovascular risk. The kinetics of Na/Li CT are modified by two types of thiol group in the membrane. In choline medium, the type 1 thiol reacts with N-ethtyl maleimide (NEM) to cause a decrease in Km and increase Vmax/Km ratio. However, in the presence of external Na or Li both the type 1 or type 2 thiols react so that both Km and Vmax are reduced. Low Km of Na/Li CT has been previously reported to be a major abnormality in diabetic nephropathy (DN) and can be used to identify diabetic patients who are at high risk for DN. A recent study showed that the type 1 thiol protein controlling the Km of Na/Li CT was a 33-kD protein and the gene for this protein is going to be cloned.*
Objective: *The authors sought to identify Na/Li CT kinetic abnormalities in Type 2 diabetes in Thai patients.*
Material and Method: *Erythrocyte Na/Li CT kinetics and their modulation by thiol proteins were measured in erythrocytes from 22 patients with Type 2 diabetes and 42 normal control subjects.*
Results: *The kinetics of Na/Li CT in untreated erythrocytes were similar. Thiol protein alkylation with NEM generally caused both Vmax and Km to fall, but caused Km to rise in erythrocytes of diabetic patients, whose native Km was low. Thus, abnormalities in the regulation of Na/Li CT by key thiol proteins were found in about one-third of subjects with Type 2 diabetes in Thailand.*
Conclusion: *Membrane abnormalities may indicate a common pathway of pathological mechanism found in essential hypertension and diabetic nephropathy and may be used as a phenotype for further genetic studies of this transporter.*

Keywords: *Cardiovascular disease, Diabetic nephropathy, Sodium lithium countertransport, Type II, Type 2 diabetes mellitus*

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There is evidence showing that diabetes and metabolic syndrome is now a global epidemic. In a large scale survey involving more than 6,600 Asian patients with type2 diabetes mellitus, it was found that 40% had micro and 20% had macroalbuminuria[1,2]. Together with the onset of diabetic chronic kidney disease, it was demonstrated that there is further disturbance of the metabolic milieu characterized by a high turnover of the oxidative pathway, anemia, acidosis and abnormal bone metabolism. All these characteristics contribute to a 5-8 fold increased risk of cardiovascular disease in patients with diabetic chronic kidney disease. Diabetic nephropathy is a condition that appears to arise from an interaction of genetic, metabolic, hemodynamic and growth factors[3,4]. An increased activity of sodium lithium counter transport (Na/Li CT) is a common finding in patients with essen-

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tial hypertension[5]. There is evidence showing that increased activity of Na/Li CT is also found in the hyperfiltration period in type1 diabetic nephropathy patients[6]. It is, therefore, hypothesized that a predisposition to essential hypertension may be the factor that leads to the development of diabetic nephropathy. Na/Li CT has a large inherited component and may be an inherited marker for an abnormality in the cell membrane. This could explain the clinical and biochemical features of diabetic nephropathy[7].

Diabetes, hypertension, chronic kidney disease and cardiovascular diseases are all known to be the major health problems in Thailand. Most therapeutic and prevention strategies so far relied on the information from the Western countries where the population is distinct from Thailand in several ways. This distinction includes environmental status, dietary habit, activities and genetic background. Thus, a search for variance of the kinetics of erythrocyte Na/Li CT in Thai diabetic patients would provide a better understanding in the pathogenesis of diabetic nephropathy.

Material and Method

Patients and normal controls

Twenty two patients with type 2 diabetic nephropathy aged between 18 and 70 years were studied. Forty two normal controls were selected from hospital staff and medical students on the basis of age and sex distribution to match type2 diabetic patients. They all had no family history of hypertension or diabetes. The details of the present study were sent to the Ethics Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University for approval. All patients and normal controls gave their informed consent before taking part in the present study.

Kinetic study of sodium-lithium countertransport

The method used was similar to that originally described by Canessa et al[8] with minor modifications. Venous blood was drawn into tubes containing lithium heparin and centrifuged for 5 minutes at 2,500g. Two mL of erythrocytes were suspended in 8 mL lithium loading solution (140 mmol/L lithium chloride, 10 mmol/L lithium carbonate, 10 mmol/L glucose, and TRIS-acetate (pH 7.5); a mixture containing 95% oxygen and 5% carbon dioxide was bubbled through the solution until the lithium carbonate dissolved) for 1.5 hours at 37°C. The erythrocytes were then washed once with $MgCl_2$ (289 $\pm$ 1 mosmol/kg) and twice with choline medium [139 mmol/L choline chloride, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 10 mmol/L TRIS-3-(N-morphalino)-propanesulphonic acid (TRIS-MOPS; pH 7.4), osmolality 290 mmol/kg]. After the final washing, the packed cell volume of the erythrocytes was measured using a micro-hematocrit and 0.2 or 0.25 mL of the packed cells was incubated in 4 mL of choline-ouabain medium (as above but containing 1 mmol/L ouabain) or 4 mL of medium with a range of sodium concentrations (1 to 150 mmol/L) made by mixing the choline medium with sodium-ouabain medium [145 mmol/L NaCl, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 10 mmol/L TRIS-MOPS (pH 7.4); osmolality 290 mmol/kg, in the presence of 1 mmol/L ouabain]. Samples were taken after incubation for 30, 60, and 90 minutes at 37°C. After centrifugation of the incubation mixtures at 2,000 g for 3 minutes, 1 mL of supernatant was mixed with 1 mL of distilled water and the lithium content measured using atomic absorption spectrophotometry (Varian: Spectraa 250 Plus) using incubation media blanks.

Michaelis constant for extracellular sodium (Km) and maximum velocity (Vmax) of sodium-lithium countertransport

The kinetic parameters Km and Vmax of Na/Li CT were determined essentially according to the method of Rutherford et al[9,10]. The Km for extracellular sodium was calculated using:

$$\text{flux rate} = Vmax - (\text{flux rate} / [Na^+]_e) / Km$$

where Vmax is the maximum reaction velocity and $[Na^+]_e$ is the extracellular concentration of sodium. The flux rate was plotted against flux/$[Na^+]_e$ and the maximum reaction velocity was determined from the intercept on the y-axis.

Statistical analysis

The values for variables that were normally distributed in all groups are given as the mean and standard error of the mean (SEM) and the probability of differences between the means were assessed using the paired or unpaired Student's *t* test where appropriated. In other cases, in non-normal distribution results are given as median and range, and Wilcoxon sign rank test for paired differences was used. Statistical significance between means were set at $p < 0.05$.

Results

The clinical characteristics of type2 diabetic patients and normal controls are shown in Table 1. There were no significant differences in age, sex, body

mass index (BMI), renal function, electrolytes, and globulin in diabetic patients compared with the normal controls. Cardiovascular risk factors including cholesterol, triglyceride, LDL and HDL were similar in both groups. The kinetics of erythrocyte Na/Li CT are shown in Table 2. Kinetic parameters of erythrocyte Na/Li CT were abnormal in subjects with diabetic nephropathy compared with non-diabetic control subjects with both low Km for external sodium and high Vmax/Km ratio reflecting ion association significantly higher. N-ethyl maleimide (NEM) decreased Km for external sodium of Na/Li CT in normal control but had no significant

Table 1. Clinical data of type2 diabetic nephropathy (DN) groups and normal controls (NC) (N = 64)

	DN (N = 22)	NC (N = 42)
Age (yrs)	47 ± 2.3	44 ± 2.5
Gender (male:female)	10:12	22:20
BMI (kg/m^2)	25.0 ± 0.18	22.9 ± 0.10
Systolic BP (mmHg)	119 ± 3*	110 ± 3
Diastolic BP (mmHg)	86 ± 2	78 ± 3
BUN (mg/dL)	22.4 ± 1.2	19.2 ± 0.5
Creatinine (mg/dL)	1.30 ± 0.09	1.12 ± 0.03
Cholesterol (mg/dL)	216 ± 11*	179 ± 5
Triglyceride (mg/dL)	102 ± 11*	100 ± 10
HDL-cholesterol (mg/dL)	54 ± 3*	65 ± 2
LDL-cholesterol (mg/dL)	119 ± 10*	107 ± 5
Uric acid (mg/dL)	7.3 ± 0.6*	6.3 ± 0.2
Sodium (mmol/L)	134.5 ± 0.4	135.8 ± 0.2
Potassium (mmol/L)	4.4 ± 0.16	4.3 ± 0.04
Chloride (mmol/L)	102.1 ± 0.8	104.2 ± 0.5
Bicarbonate (mmol/L)	25.2 ± 0.9	23.6 ± 0.5
Albumin (mg/dL)	4.2 ± 0.1*	4.1 ± 0.1
Globulin (mg/dL)	2.9 ± 0.1	3.1 ± 0.1
Hb (g/dL)	14 ± 3	15 ± 5

Values are mean ± SEM, * $p < 0.05$ compared with normal controls

Table 2. Kinetic parameters of erythrocyte Na/Li countertransport in type2 diabetic nephropathy (DN) group and normal controls (NC) (N = 64)

	DN (N = 22)	Normal Control (N = 42)
Vmax		
Untreated	0.50 ± 0.04	0.48 ± 0.03
NEM/choline	0.52 ± 0.03	0.44 ± 0.02*
NEM/sodium	0.30 ± 0.01*	0.26 ± 0.02*
Km		
Untreated	60 ± 3	86 ± 2
NEM/choline	72 ± 2*	60 ± 4*
NEM/sodium	56 ± 4*	58 ± 3*
Vmax/Km		
Untreated	8.33 ± 0.13	5.58 ± 0.15
NEM/choline	7.22 ± 0.15	7.73 ± 0.05*
NEM/sodium	5.36 ± 0.25	4.48 ± 0.06*

Units: Vmax, mmol Li $(hr \times l\ rbc)^{-1}$, Km, mmol Na l^{-1}
Values are mean ± SEM
*$p < 0.05$ for untreated vs NEM treatment

effect on the Km for external sodium in diabetic patients.

Discussion

These data suggest that diabetic nephropathy in Thai patients is associated with abnormal characteristics of erythrocyte Na/Li CT. Erythrocyte Na/Li CT is an obligatory equimolar exchange of intracellular sodium or lithium ions with extracellular sodium or lithium ions and is a counter transport that does not require the presence of cellular ATP, potassium or HCO_3^- ion[11]. This transporter is insensitive to ouabain, oligomycin and amiloride, a sodium-proton exchanger inhibitor. The physiological functions of Na/Li CT can be partially, approximately 50%, inhibited by the thiol-alkylating agent, NEM[12]. By critical analysis in the kinetics of Na/Li CT, Thomas et al have found that this transporter can be modulated by at least 2 types of thiol proteins as mentioned previously. The kinetics of Na/Li CT follow Michaelis-Menten equation and its activity can be measured by determining the difference between the rates of lithium efflux into sodium-rich (150 mmol/L) and sodium-free (0 mmol/L) media. Several studies suggested that the standard Na/Li CT activity, under the conditions of the standard assay, depends on two independent kinetic parameters, the maximum velocity (Vmax) and the sodium affinity (Km) at the external site of the countertransporter[13]. Km is the rate constant for ion association and Vmax is the rate constant for ion translocation. Recent models have demonstrated that Km is not an ideal rate constant because it may be affected by several rate constants of the transport process including the rate constant for the translocation of ions across the membrane (Vmax). An alternative rate constant for ion association can be derived from the Michaelis-Menten equation:

$$v = \frac{Vmax\ [Na^+]_e}{[Na^+]_e + Km}$$

When $[Na^+]_e$ is negligibly small in relation to Km, there is a negligibly small amount of external sodium transport complex and

$$v = (Vmax/Km) \cdot [Na^+]_e$$

Vmax/Km is then effectively the first-order association rate constant for the association of external $[Na^+]_e$ with the unloaded transporter. By critical analysis of the kinetic characteristics of Na/Li CT, it was found that several hypertensive-related diseases, such as essential hypertension, diabetic nephropathy, and Autosomal Dominant Polycystic Kidney Disease (ADPKD) are in the group characterized by a decrease in Km and a high Vmax/Km ratio[14]. The present study has clearly shown that the abnormality of erythrocyte Na/Li CT, in terms of low Km for external sodium and high Vmax/Km ratio, were also found in Thai diabetic patients. NEM is a thiol group alkylation and can modulate the membrane cytoskeletal proteins by thiol group alkylation. Several evidences have demonstrated that thiol proteins might be an important group of proteins that play a key role in the pathogenesis of several hypertensive-related diseases, such as essential hypertension, diabetic nephropathy and ADPKD.

Na/Li CT is a cation transporter on membranes of red blood cells. It becomes clear that the activity of erythrocyte Na/Li CT can be used as a marker to identify patients with high cardiovascular risks[15]. Elevated erythrocyte Na/Li CT activity identifies a group of essential hypertensive patients with a genetic predisposition to hypertension and cardiovascular disease[16,17]. Subsequent studies have been demonstrated that its high activity was also found in diabetic nephropathy patients[18,19]. By kinetic study, it was found that the Km for external sodium of erythrocyte Na/Li CT was low in patients with essential hypertension and diabetic nephropathy[9,10,20]. Recently, it has been clearly shown that the kinetics of Na/Li CT are also abnormal in other cardiovascular-related diseases such as dyslipidemia, pregnancy related-hypertension[21], and ADPKD[22]. Thomas et al have demonstrated that the kinetics of Na/Li CT are controlled by at least 2 types of thiol proteins[23]. The type1 thiol group controls the Km for external sodium and the type 2 thiol group controls the Vmax of this transporter. Thiol proteins generally play a major role in the cellular oxidative pathway[24]. Alterations in the oxidative pathway may lead to atherogenesis. By molecular biology approach, it has been recently demonstrated that the type1 thiol protein that modulate the Km of erythrocyte Na/Li CT has a molecular weight of 33 kD and its gene is going to be cloned in the near future[25]. The result of the present study indicates that the specific group of thiol proteins, which is an inheritance protein for hypertensive-related diseases, was abnormal in Thai diabetic nephropathy patients. Study of the kinetics parameters of erythrocyte Na/Li CT, including thiol group modulation, suggests that increased ion association, Vmax/Km ratio may represent the inherited defect of diabetic nephropathy patients. This abnormality of this transporter might be used to identify

subjects who have a high risk for diabetic nephropathy.

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ความผิดปกติทางจลศาสตร์ของโสเดียม ลิเธียม เคาน์เตอร์ทรานสปอร์ตในผู้ป่วยไตพิการเหตุเบาหวานในประเทศไทย

เกรียงศักดิ์ วารีแสงทิพย์, วีรวัฒน์ พานทองดี, ชัยรัตน์ ฉายากุล, วรรณี นิธิยานันท์, ลีนา องอาจยุทธ

ในปัจจุบันมีหลักฐานยืนยันว่า โสเดียม ลิเธียม เคาน์เตอร์ทรานสปอร์ต (Na/Li CT) สามารถถ่ายทอดทางกรรมพันธุ์ในผู้ป่วยที่เป็นโรคความดันเลือดสูง และภาวะไตพิการเหตุเบาหวาน Na/Li CT เป็นตัวบ่งชี้ถึงผู้ที่มีอัตราเสี่ยงต่อการเกิดโรคระบบหลอดเลือดและหัวใจ ภาวะจลศาสตร์ของ Na/Li CT ถูกควบคุมด้วย thiol protein 2 กลุ่ม โดย thiol protein 1 ควบคุมอัตราการจับตัวของ ทรานสปอร์ตเตอร์ กับ โสเดียม อิออน [Na^+] และ thiol protein 2 ควบคุมอัตราการเคลื่อนตัวของ ทรานสปอร์ตเตอร์ ที่จับ [Na^+] แล้วผ่านเซลล์เมมเบรนเข้าเซลล์ มีงานวิจัยศึกษา พบว่า Michaelis constant for extracellular sodium (Km) ของ Na/Li CT มีค่าต่ำลงในผู้ป่วย ไตพิการเหตุเบาหวาน และพบว่า thiol protein 1 ที่ควบคุม Km และโปรตีนที่มีน้ำหนักโมเลกุล 33-KD และกำลังศึกษาวิจัยถึงโครงสร้างของโปรตีนนี้เพื่อนำไปสู่การโคลนยีนส์

ในการศึกษานี้ผู้วิจัยมีวัตถุประสงค์ศึกษาจลศาสตร์ของ Na/Li CT ในผู้ป่วยเบาหวานที่มีภาวะแทรกซ้อนทางไต จำนวน 22 ราย เปรียบเทียบกับประชากรปกติจำนวน 42 ราย ผลการศึกษาพบว่า ค่า Km ของ Na/Li CT ต่ำกว่าประชากรปกติอย่างมีนัยสำคัญ ในการศึกษาในประชากรของประเทศทางตะวันตก พบภาวะ Km ต่ำ ได้ในผู้ป่วยที่เป็นโรคความดันเลือดสูงและไตพิการเหตุเบาหวาน ผลการศึกษาวิจัยนี้สนับสนุนภาวะ Km ต่ำในผู้ป่วยไตพิการเหตุเบาหวาน ในประชากรไทย ซึ่งจะมีประโยชน์ในการศึกษาถึงกลไกการเกิดภาวะแทรกซ้อนทางไตในผู้ป่วยเบาหวานในประเทศไทยในลำดับต่อไป