ผลของวานาเดต และอาหารที่มีโปแตสเซียมต่ำต่อระดับโปรตีน
H,K-ATPase และ Na,K-ATPase ที่ไตในหนูขาว

นางสาว รัตนาภรณ์ จีระวัฒนะ

EFFECTS OF VANADATE AND LOW POTASSIUM DIET
ON RENAL H,K-ATPase AND Na,K-ATPase
PROTEIN EXPRESSION IN RAT

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รัตนาภรณ์ จีระวัฒนะ: ผลของวานาเดต และ อาหารที่มีโปแตสเซียมต่ำต่อระดับโปรตีน H,K-ATPase และ Na,K-ATPase ที่ไตในหนูขาว (EFFECTS OF VANADATE AND LOW POTASSIUM DIET ON RENAL H,K-ATPase AND Na,K-ATPase PROTEIN EXPRESSION IN RAT) อ.ที่ปรึกษา: ผศ.ดร. สมจิตร เอี่ยมอ่อง, อ.ที่ปรึกษาร่วม: ผศ.พญ. วิภาวี กิตติโกวิท 82 หน้า ISBN 974-17-6682-3

การวิจัยครั้งนี้ เป็นการศึกษาผลของวานาเดต และอาหารที่มีโปแตสเซียมต่ำต่อระดับ Na,K-ATPase และ H,K-ATPase โปรตีนในไตหนูขาว โดยทำการทดลองในหนูแรทเพศผู้ พันธุ์วิสต้า ซึ่งแบ่งออกเป็นสองกลุ่มใหญ่ๆ คือ กลุ่มที่ได้รับน้ำเกลือ (NSS) หรือ ได้รับวานาเดต (V; ปริมาณ 5 มก./น้ำหนักตัว 1 กก.) ฉีดทางช่องท้อง สัตว์ทดลองในแต่ละกลุ่มจะได้รับอาหาร ที่มีโปแตสเซียมปกติ (NK) หรือ ที่มีโปแตสเซียมต่ำ (LK) เป็นเวลา 10 วัน เมื่อครบกำหนด การทดลอง ทำการเก็บตัวอย่างปัสสาวะและเลือด เพื่อตรวจหาระดับวานาเดียม อิเล็กโทรไลต์ ยูเรียไนโตรเจน ครีเอตินิน (Cr) และ C_{cr} เก็บตัวอย่างเนื้อไตเพื่อตรวจวัด Na,K-ATPase และ H,K-ATPase โปรตีน รวมทั้งวิเคราะห์ระดับวานาเดียมในเนื้อไต ผลการทดลองพบว่าการให้ วานาเดตเป็นเวลา 10 วัน เพิ่มระดับวานาเดียมในเลือด ปัสสาวะ และเนื้อไต ทั้งในกลุ่ม NK และ LK อย่างมีนัยสำคัญทางสถิติ ส่วนผล immunohistochemistry พบว่าระดับ Na,K-ATPase โปรตีนที่บริเวณหลอดไตส่วนท้ายและหลอดไตรวม   มีค่ามากกว่าส่วนอื่นๆของหลอดไต ทั้งบริเวณ cortex และ medulla ค่าระดับดังกล่าวนี้จะเพิ่มขึ้นโดย LK แต่จะลดลงเมื่อได้รับ V พบว่า LK ไม่สามารถเพิ่มระดับ Na,K-ATPase ในสัตว์ทดลองที่ได้รับ V ร่วมด้วย ส่วน H,K-ATPase โปรตีนที่หลอดไตรวมบริเวณ cortex มีระดับเพิ่มขึ้นเช่นกัน ทั้ง α1 และ α2 subunit ใน ภาวะ LK แต่ที่บริเวณ medulla นั้น LK เพิ่มเฉพาะ α1 โปรตีนเพียงเล็กน้อย โดยที่ไม่มี expression   ของ α2 โปรตีนในบริเวณนี้ การให้ V ไม่มีผลต่อระดับ H,K-ATPase โปรตีน ทั้ง α1 และ α2   นอกจากนี้ เมื่อสัตว์ทดลองที่ได้รับ V ร่วมกับ LK จะมีค่าระดับโปแตสเซียม และคลอไรด์ในเลือดลดลงอย่างต่อเนื่อง รวมทั้งเกิดภาวะ azotemia และ natriuresis ส่วนกลุ่ม LK ที่ได้รับ NSS หรือ V มีค่า pCO_2 ในเลือดลดลง แต่ค่า pH ยังคงปกติ สัตว์ทดลองที่ได้รับ V ร่วมกับ LK มีค่าสัดส่วนการขับทิ้งของโปแตสเซียม คลอไรด์ และของไบคาร์บอเนตเพิ่มขึ้น สัตว์ทดลองทุกกลุ่ม มีค่าอัตราการขับปัสสาวะที่ใกล้เคียงกัน

ผลการศึกษาครั้งนี้ เป็นรายงานชิ้นแรกที่แสดงว่า LK ไม่สามารถเพิ่มระดับ Na,K-ATPase โปรตีนที่ไตที่มีค่าลดลงจากการได้รับ V ส่วนระดับของ H,K-ATPase โปรตีนที่ไตนั้น พบว่ามีค่าไม่เปลี่ยนแปลงเมื่อให้ V อย่างไรก็ตาม ภาวะ LK ยังคงเป็นตัวกระตุ้นที่สำคัญในการ เพิ่มระดับ H,K-ATPase โปรตีน ทั้ง α1 และ α2 การให้ V ร่วมกับ LK สามารถเปลี่ยนแปลงค่า metabolic parameter และค่าการทำงานของไต ได้มากกว่าการให้ V หรือ LK เพียงอย่างเดียว

หลักสูตร..ลายมือชื่อนิสิต...….....

สาขาวิชา ....ลายมือชื่ออาจารย์ที่ปรึกษา ....

ปีการศึกษา ....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม ....…..................

This study was conducted to investigate the effect of vanadate and low potassium diet on renal Na,K-ATPase and H,K-ATPase protein expression. Male Wistar rats were divided into two main groups: normal saline solution (NSS) or vanadate (V; 5 mg/kg. BW) injection. In each group, the animals were received either normal-potassium (NK) or low-potassium (LK) diet. The treatments were performed for 10 days. On each experimental due date, 24-hr urine and blood samples were collected. The serum was stored at -80°C until use for measurement of vanadium, electrolytes, blood urea nitrogen (BUN), creatinine (Cr), and Cr clearance (C_{cr}). The kidneys were removed and fixed for measurement of Na,K-ATPase as well as H,K-ATPase (α1 and α2 isoforms) protein expression. Some renal tissue samples also were determined for vanadium concentration. Ten days of vanadate administration significantly increased vanadium levels in serum, urine, and renal tissues in both NK and LK groups. By immunohistochemistry, the expression of renal Na,K-ATPase protein showed the main staining in distal tubule and collecting duct both in cortex and medulla. The expression was increased by LK but was reduced by V. LK in V treated rats could not restore the expression. For H,K-ATPase, in cortex, LK could enhance the protein expression of both α1- and α2-subunit at luminal membrane of collecting duct. In medulla, LK slightly increased α1-isofrom protein expression, whereas no expression of α2 was noted. Vanadate did not affect on H,K-ATPase protein expression both α1 and α2. Administration of both V and LK caused a progressive hypokalemia as well as induced azotemia and natriuresis. LK in either NSS or V treated rats significantly reduced blood pCO_2 while blood pH was remained normal. Treatment of V in LK animals increased fractional excretion of potassium, chloride, and bicarbonate. All experimental rats showed no significant changes in urine flow rate.

The present data are the first evidence showing that LK could not restore the suppressive effect of V on renal Na,K-ATPase protein expression. Vanadate has no influence on renal H,K-ATPase protein expression. However, LK still plays an important stimulus to increase H,K-ATPase protein expression both α1 and α2 isoforms. Combination treatment of V and LK caused more profound alterations in metabolic parameter as well as renal function than those subjected to either V or LK alone.

Department………………………… **Student's signature**……………………………..

Field of study……………...……….. **Advisor's signature**…………………………...

Academic year……………………**Co- advisor's signature**………………………

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LIST OF ABBREVIATIONS

ABG	Arterial blood gas
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BUN	Blood urea nitrogen
BW	Body weight
C_{Cr}	Creatinine clearance
Cr	Creatinine
°C	Degree celsius
DAB	3, 3'- diamino-benzidine
FE	Fractional excretion
g	Gram
hr	Hour
H_2O_2	Hydrogen peroxide
ip	Intraperitoneal
IHC	Immunohistochemistry
Kg	Kilogram
KH_2PO_4	Potassium di-hydrogen phosphate
LK	Low potassium
M	Molar
MC	Metabolic cage
mEq	Milliequivalent
µl	Microlitre
Na_2HPO_4	Di-sodium hydrogen phosphate
Na_3VO_4	Sodium orthovanadate

LIST OF ABBREVIATIONS (cont.)

NHS	Normal horse serum
NK	Normal potassium
NSS	Normal saline solution
rpm	Revolution per minute
S	Serum
V	Vanadate

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Vanadate is an oxyanion derivative of the ubiquitous trace metal, vanadium, which occurs in various concentrations in soil, water, air, plants, animal and human tissues (WHO, 1988). Exposure to vanadium is of concern due to an increased concentration near industrial operations. Vanadium occurred as a by-product of petroleum and metallurgical refinement, and its subsequent accumulation in the environment (Phillips et al., 1983).

Human could intake vanadium by eating certain foods, breathing air near industries (Phillips et al., 1983). Exposure to high levels of vanadium can cause harmful health effects. At higher intakes, it could accumulate in body tissue such as liver, kidneys, and bones, while the smallest amount is found in the brain (French and Jones, 1992). Interestingly, the highest accumulation of vanadium is found in renal tissue (Parker and Sharma, 1978; Phillips et al., 1983) and is principally eliminated in the urine. Therefore, the kidneys would represent a major site of toxic and pharmacological actions of vanadium (Grantham et al., 1980).

Cantley et al. (1977) reported that vanadate is a potent inhibitor of Na,K-ATPase. Grantham et al. (1979) also showed that the Na,K-ATPase activity in renal tissue homogenate was reduced by vanadate in a dose dependent response. Furthermore, Midendorf et al. investigated the effect of intravenous injection of sodium orthovanadate (Na_3VO_4) in conscious rats, and found that vanadate increased water and sodium excretions in a dose dependent manner. They suggested that vanadate might inhibit renal

Na,K-ATPase. In addition, Boscolo et al. (1994) used the histochemical method to detect Na,K-ATPase activity in rats treated with vanadate. The findings showed that the rats exposed to vanadate had a reduction in protein expression of Na,K-ATPase.

Chronic vanadate administration inhibited H,K-ATPase and Na,K-ATPase activity in both cortical and medullary collecting tubule (Dafnis et al., 1992). Vanadate could impair the expected increase in urinary net acid excretion in acute acid-loaded animals (Eiam-Ong et al., 1996). The impairment of urinary acidification induced by vanadate may relate to its inhibitory effect on H,K-ATPase and Na,K-ATPase.

It has been known that a low potassium diet intake results in an increase in potassium reabsorption by the collecting tubule (Imbert et al., 1987). During potassium defeciency, H,K-ATPase activity (Doucet and Marsy, 1987; Eiam-Ong et al., 1993; Ibrahim et al., 1995) and Na,K-ATPase activity were enhanced (Eiam-Ong et al., 1993), and also the reabsorption of potassium is increased.

Vanadate suppresses while a low potassium diet stimulates both H,K-ATPase and Na,K-ATPase activities. Heretofore, there have been no available data regarding the simultaneous effect of vanadate and low potassium diet on renal H,K-ATPase and Na,K-ATPase protein expressions. Therefore, this study was conducted to examine this regard.

CHAPTER II

THEORY AND LITERATURE REVIEW

Vanadium

Vanadium (V) is a metallic element in a number of the Vb group of transition metal in the periodic table. Vanadium is the 21st most abundant element in the earth's crust, and would occur in nature as a white to gray metal and molecular weight is 50.942. It was discovered by the Swedish chemist Nils Gabriel Sefstron in 1830 and named after the Scandinavian goddess "Freya vanadis" (Dafnis and Sabatini, 1994; Grantham, 1980).

Vanadium is widespread with various concentrations in all environments including rock, soil, water, air, plants, and animal tissues (Barceloux, 1999; WHO, 1988). Vanadium is used widely in industrial processes including the production of special vanadium-iron steels, in the production of hard metals and temperature-resistant alloys, in glass industry and in the production of electronics and ceramics (Phillip, Nechay, and Heidlbagh, 1983). These processes could release large quantities of vanadium into the environment (Jandhyala and Hom, 1983; Lener, 1998). Exposure to vanadium is of concern due to an increased concentration near industrial operations. Vanadium occurred as a by-product of petroleum and metallurgical refinement, and its subsequent accumulation in the environment (Phillips et al., 1983).

Chemistry of Vanadium Compounds

Vanadate is a salt of vanadium compounds. Vanadium possess 2 naturally occurring isotopes, 50V and 51V (Clark, 1975). It could form oxidation states of –1, 0, +1, +2, +3, +4, +5, of which the state of +4, and +5 are the most stable. In biological systems, vanadium is present in the extracellular fluid principally in the +5 oxidation state (vo_4^-, vanadate). Intracellularly, it is reduced to the +4 oxidation state (vo^{2+}, vanadyl) (Nechay, 1984; Phillips et al., 1983).

In the body, vanadium can undergo changing in oxidation state and it can also form chemical complexes with blood proteins (Breuch et al., 1984; Neves et al., 1998; Thomson et al., 1998). Harris et al. (1984) followed the distribution of vanadium in blood, cell, and plasma of dog injected intravenously with radio labeled vanadyl chloride (+4 oxidation state) or ammonium vanadate (+5 oxidation state). A significant fraction of the vanadium was associated with red blood cell and 77% of the plasma vanadium was eventually bound to serum transferrin. The authors suggested that there is an interconversion of vanadyl and vanadate in the blood, probably with the oxidation of the vanadyl transferrin complex taking place in the plasma whereas the reduction of vanadate to vanadyl ions occurring in red blood cell.

Distribution of Vanadium

Vanadium (V) is a pervasive element of biological systems, being widely distributed across the food supply, usually in the form of vanadyl (+4 oxidation state) or   vanadate (+5 oxidation state) (Nechay, 1984).

Particularly rich in vanadium are mushrooms, parsley, dill and black pepper. Fresh fruits, vegetables, and beverages constitute some of the poorer sources of vanadium with seafood and cereals being of intermediate quality. Concentration of vanadium tend to be higher in processed than unprocessed foods (French and Jones, 1992; Myron, 1977). For example, dairy milk has an average value of 1.1 µg/kg of vanadium whereas powdered milk shows a value as high as 25 µg/kg (WHO, 1988). Vanadium content in diets from different parts of the world does not differ much, the mean values being in the range of 20.00 to 69.4 µg/kg dry weight. Thus, considering consumption of about 500 g (dry weight) of the total diet, daily vanadium intake can be estimated as10 to 20 µg/day (Byrne and Kosta, 1978). The average mean of human serum concentration of vanadium is about 0.35 ng/ml (Nechay, 1986).

Vanadium absorption in the gastrointestinal tract is low, or moderate when inhaled, and high when injected (WHO, 1988). The main route of excretion of absorbed vanadium is through the kidney. Vanadium levels in urine are of the order of 0.1 to 0.2 µg/L or about 12% of the amount intake. The ratio of amounts eliminated in the urine and feces is 5:1 (Talvite and Wagner, 1954). At higher intakes, vanadium could accumulate in body tissue such as liver, kidney, and bone whereas the smallest amounts are found in the brain (French and Jones, 1992). Interestingly, the highest accumulation is found in renal tissue (Adchi, 2000).

In 1978, Parker et al. studied the distribution and effect of selected vanadium salts in male rats chronically treated with continuous ingestion of 5 or 50 ppm of vanadate, as vanadyl sulfate or sodium orthovanadate for 3 months. The results from the higher dose showed increased levels of vanadium in all tissue studied (blood, kidney, liver, bone, muscle, and

digestive tract). Moreover, at the higher dose, sodium orthovanadate appeared to show a higher accumulation in renal tissue than that of vanadyl sulfate. It was shown that vanadium accumulation in tissue is directly related to the dose administered (Adchi, 2000; Parker et al., 1978).

Toxicity of Vanadium

The toxicity of vanadium has been reported by many researchers(Jandhyala and Hom, 1983; Phillips et al., 1983; Nechay et al., 1986; Russanov, 1994; Liuz, 2002). In general, vanadium can enter the environment from natural sources and from the burning of fuel oils, especially crude oil. It can stay in air, water, and soil for a long time. Vanadium does not dissolve well in water, but it combines with other particles and adheres to soil sediments. Human intake vanadium by eating certain foods, breathing air near industries that burn fuel oil or coal, working in industries that make products containing it, or drinking contaminated water near waste site or landfills containing vanadium (WHO, 1988). In Thailand, the northeastern has been known to be the vanadium-rich environment area. Vanadium has been shown to occur in the urine of 30% of the people in this region studied (Sitprija et al., 1990).

Exposure to high levels of vanadium can cause harmful health effects (WHO, 1988). The major effects from breathing high levels of vanadium are on the lungs, throat, and eyes. Workers who breath in vanadium for short or long periods, sometime have lung irritation, coughing, bronchitis, bronchospasm, chest pain, runny nose, and sore throat. Likewise, vanadium has been shown to produce gastrointestinal distress, fatigue, cardiac palpitation, and kidney damage. These toxic effects have also been linked with industrial exposure (Jandhyala and Hom,

1983; Phillips et al., 1983; Nechay et al., 1986). These effects stop soon after they did not breath the contaminated air (Zenz et al. 1962). Vanadium has been demonstrated to produce other physiological effects such as disturbance of the central nervous and cardiovascular changes, as well as metabolic alteration of experimental animal (Phillips et al., 1983). In kidney, vanadate enchances lipid peroxidation (Donaldson, 1984), induces kidney fibrosis (Al-Bayati, 2002), and decreases the activity of antioxidation enzyme (Russanov, 1994; Liuz, 2002). The toxicity of vanadium to human through industrial exposure increases with the oxidation state or vanadium compound, with the 5+oxidation state (vanadate) being the most toxic. Similar effects have been observed in animal studies. Vanadium also interferes with a multitude of biochemical processes, by penetrating the blood-brain and placental barriers and presenting in breast milk and saliva (Hackett and Kelman, 1983). Moreover, vanadium accumulates in renal tissue to a large extent and is principally eliminated in the urine. Thus, the kidneys may represent a major site of toxic and pharmacological action of vanadium.

Effects of Vanadate on Renal Function

Trace amounts of vanadium can be detected in most mammalian tissue; the highest concentrations are often found in the kidney, especially in the renal cortex. It is excreted in the urine and is accumulated by renal cells during the course of its elimination (Bogden et al., 1982). When infused intravenously or intrarenally into rats, it also causes a remarkable natriuresis and diuresis (Day et al., 1980; Hatfield and Churchill, 1981; Higashi and Bello-Reuss, 1980; Westenfelder et al., 1981). Since profound diuresis is seen even when the GFR is unchanged, vanadate must inhibit

sodium and water absorption in renal tubules rather than cause natriuresis and diuresis by processes tied only to hemodynamic alterations. In rats, vanadate inhibits primarily proximal tubules sodium, chloride, water, bicarbonate and glucose reabsorptions, and p-aminohippurate (PAH) secretion (Smith et al., 1982). Vanadate depresses free water formation, sodium reabsorption, and potassium secretion along the ascending limb of Henle's loop, resulting in urinary concentration defect (Edwards and Grantham, 1983; Higashi and Bello-Reuss, 1980; Westenfelder et al., 1981). It has been suggested that fractional reabsorption of sodium could be decreased in proximal tubule, but the excess sodium and water reabsorption occurred in distal sites of the nephron (López-Novoa et al., 1982). The mechanism of the renal tubular functions of vanadate would be a direct result of the actions renal tubular cells ATPase system or be indirectly interfering hormone to lodge principally in the renal tubules. It has been suggested that vanadate inhibits Na,K-ATPase and Ca-ATPase in peritubular membrane and phosphotransferase and phosphohydrolase reaction but activates adenylated cyclase activity (Nechay et al., 1986).

Moreover, it has been shown that vanadate also inhibits H,K-ATPase in distal nephron segments (Doucet and Marsy, 1987; Gary and Narang, 1988) and H^+-ATPase in turtle bladder (Youmans and Barry, 1989). In addition, it can inhibit other enzymes such as an alkaline phosphatase, phosphotyrosyl protein phosphatase, the contractile protein ATPase, dynein and ect. (Nechay et al., 1986). In 1986, Nechay suggested that vanadate in living animals causes diverse effects in different organ systems, probably because vanadate inhibits a variety of phosphatase enzymes including Na,K-ATPase and H,K-ATPase; while the vanadyl form stimulates adenylate cyclase.

Sodium – Potassium Adenosine Triphosphatase (Na,K-ATPase) and the Kidney

The Na,K-ATPase enzyme is a multimeric enzyme, transmembrane-protein-spanning domain of which crosses the cytoplasmic surface of the cell membrane. In the process, it translocates sodium from the intracellular cytoplasm to the extracellular fluid and moves potassium in the reverse direction. In a single cycle, the enzyme undergoes serial phosphoryl transfers and conformational changes, thus hydrolyzing 1 molecule of ATP in exchange for 3 intracellular sodium ions and 2 extracellular potassium ions (Ganong, 2003). The activity of the pump is dependent on the activity of these 2 ions, and as with many transporters, its interaction with intracellular sodium is the most important.

The enzyme occurs in 2 comformation, E1 and E2 which are characterized by the origin of the phosphorus group bound to the enzyme. In the E1 conformation, the phosphorus group originated from the intracellular hydrolysis of ATP as the enzyme releases bound sodium into the extracellular space. In the E2 conformation, phosphorylation occurs from the free intracellular phosphorus pool through a reaction called back door phosphorylation, here the enzyme accepts 2 potassium from the extracellular side is stabilized (Grantham, 1980).

Na,K-ATPase, located principally in the basolateral cell membranes in different tissues, at least in part, in renal vasculature and all renal tubular segments which are found in the highest concentration in the distal convoluted tubule, intermediate in proximal convoluted tubule and lower in the straight portion of proximal tubule and in collecting duct (Figure A) (Garg, Knepper, and Burg, 1981; Katz, Doucet, and Morel, 1979; Schmidt and Dubach, 1969).
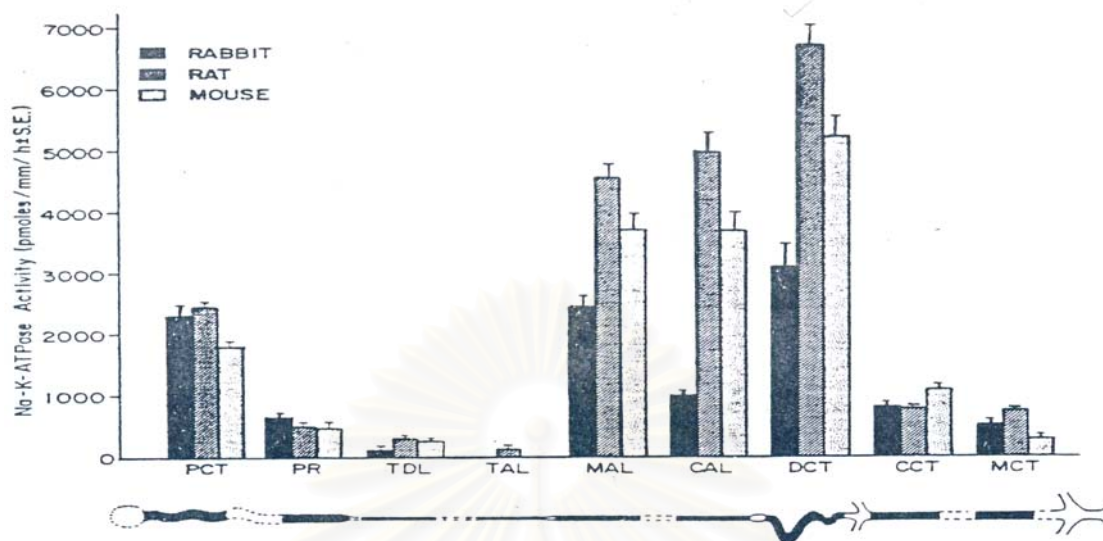
Figure A   Na,K-ATPase activity profile in the rabbit, rat, and mouse nephron. PCT = proximal convoluted tubule; PR = pars recta; TDL = thin descending limb; TAL = thin ascending limb; MAL = medullary thick ascending limb; CAL = cortical thick ascending limb; DCT = distal convoluted tubule; CCT = cortical collecting tubule; MCT = outer medullary collecting tubule (Katz, 1979).

The pump generates a gradient for sodium between cytoplasm and extracellular fluid (both urine and blood) that favors diffusion of sodium ion into the cells from both the luminal and basolateral surfaces. There may be some sodium reabsorbed passively in conjunction with transtubule gradients of other molecule into cells. Thus, Na,K-ATPase provides the energy for secondary active transport processes utilizing the potential energy stored in the electrochemical gradient (Figure B).

The Na,K-ATPase has been playing a major role in trans-membranous and transepithelial transport processes which serves a wide range of important functions, mediately transcellular of inorganic and organic compounds by the inward sodium electrochemical gradient (Grantham, 1980).
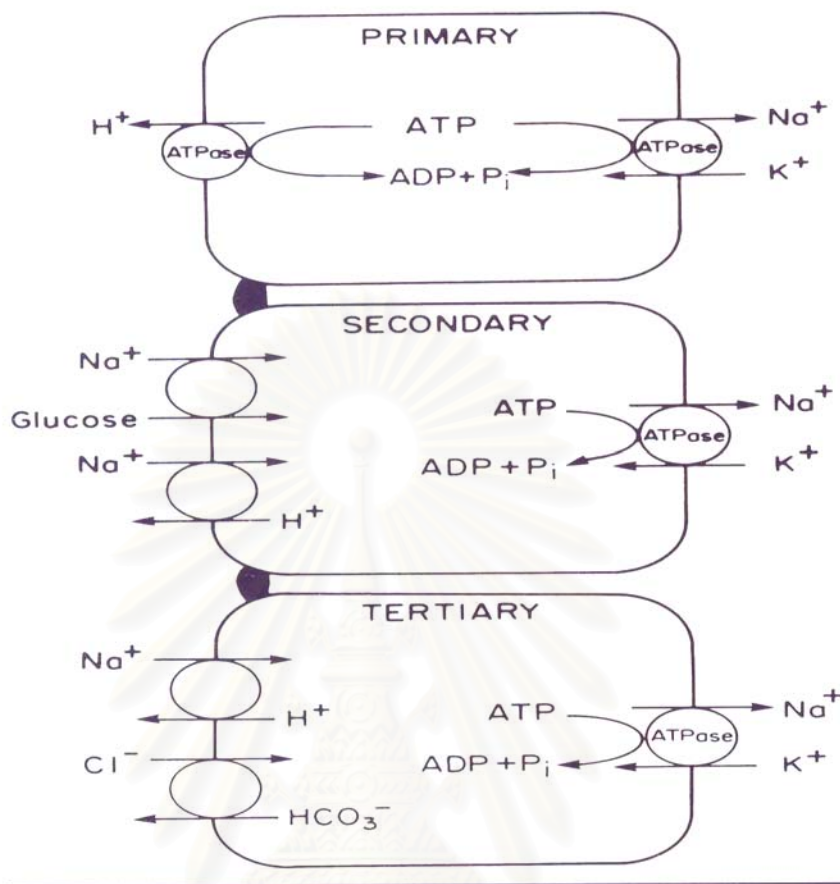
Figure B Na,K-ATPase provides the energy for secondary active transport processes utilizing the potential energy stored in the electrochemical gradient (Grantham, 1980).
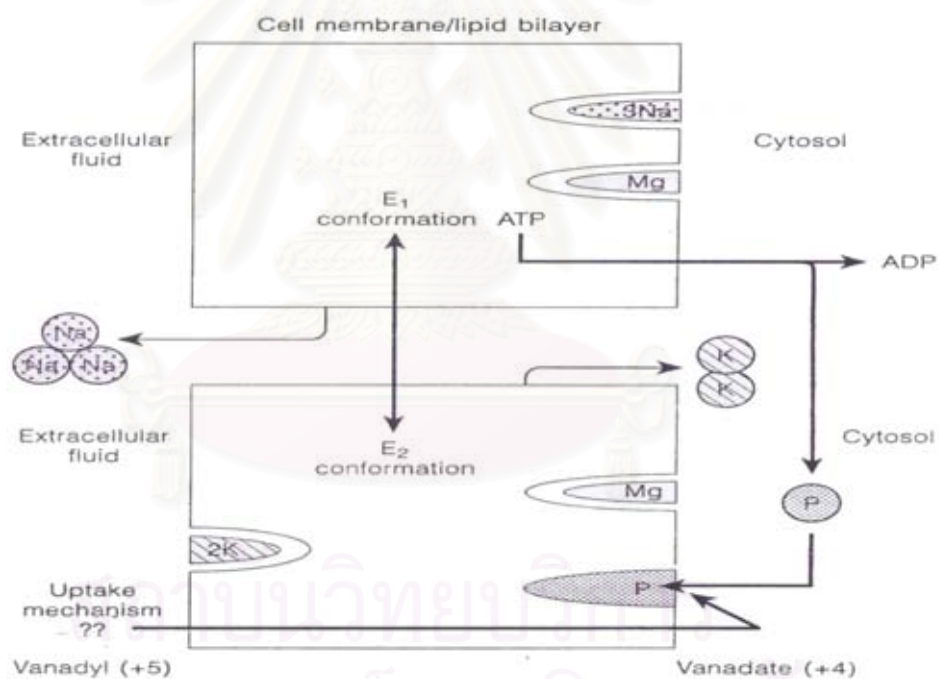
Effect of Vanadate on Na,K-ATPase

Vanadate is mainly excreted by the kidneys (Dafnis et al., 1992; Parker and shorma, 1978; WHO, 1988). In the isolate perfused rat kidney, the clearance of vanadate about one third the clearance of inulin (Kumar and Order, 1980). Accumulation of the element occurs in the kidney and its distribution appears to vary according to the route and duration of administration. Chronic vanadate treatment results in significant accumulation of vanadate in the kidneys (Domingo et al., 1991; Parker and Sharma, 1978; Mongold et al., 1990); however, most of the accumulated vanadium is likely bound to small peptides or macromolecules in the form of vanadyl and thus is not available as vanadate, a more potent inhibitor of ATPase (Phillips et al., 1983). Cantley et al. (1977) have reported that vanadate is one of the most potent known inhibitors of Na,K-ATPase. In 1980, Midendorf et al. investigated the effect of intravenous injection of sodium orthovanadate (Na_3VO_4) in conscious rats in order to determine renal function. They found that the rate of 10, 20, 30 $\mu M/$ kg/ hr of vanadate increase water and sodium excretion in dose dependent manner. They suggested that vanadate may inhibit renal Na,K-ATPase. This was unmasked by Grantham et al. (1979). They showed that the enzyme activity in renal tissue homogenate is reduced by vanadate in a dose dependent response as well. In addition, Boscolo et al. (1994) used the histochemical method to detect Na,K-ATPase activity in rats treated with vanadate. The findings showed that the rats exposed to vanadate have a reduction in protein expression of Na,K-ATPase. Moreover, in 1992, Dafnis et al. demonstrated that this enzyme activity also diminishes in collecting tubules both cortical and medullary when the animals received vanadate

intraperitonealy for 10 days. The accumulation of vanadate in kidney causes natriuresis via an inhibition of renal Na,K-ATPase activity.

The mechanism of vanadate that inhibits Na,K-ATPase enzyme occurs at the cytoplasmic side, thus it must first enter the cell where it combines with the phosphorus site of the enzyme to stabilize the pump in the E2 configuration. This progressively slows the rates of the conformational change to E1, and then enzyme inhibition occurs (Figure C) (Dafnis and Sabatini, 1994; Grantham, 1980; Huang and Askari, 1981).

Figure C Schematic representation of the effect of vanadate on the Na,K-ATPase enzyme. Vanadate inhibits Na,K-ATPase by slowing the E2-E1 transformation of the enzyme by combining with phosphate on the cytoplasmic surface of the cell. The mechanism for transport of vanadate into the cell is not known (Grantham, 1980).

Hydrogen- Potassium Adenosine Triphosphatase (H,K-ATPase) and the Kidney

H,K-ATPase has been first described in frog gastric microsomes and in the mammalian colon (Ganser and Forte, 1975; Lee et al., 1974). This enzyme is involved in the gastric acid secretion (Gustin and Goodman, 1981). H,K-ATPase expresses along the length of the late distal tubule and collecting duct contributes to urinary acidification and to potassium (K) reabsorbtion (Doucet and Marsy, 1987; Grary and Narany, 1988; Kone, 1996).

H,K-ATPase exchanges two cations (H^+ and K^+) and therefore is electroneutral and not influenced by membrane voltage. The H,K-ATPase in the distal nephron appears to involve at least two isoforms-gastric H,K-ATPase (also called HK-α1 or HKg), identical to that in gastric parietal cells (Mathews et al., 1995; Rabon et al., 1985). The second isoform is colonic H,K-ATPase (also called HK-α2 or HKc), indentical to that originally isolated from the colon (Caviston et al., 1999; Crowson and Shull, 1992). Both HK-α1 and HK-α2 have been found in the rat (Chares, Wingo, and Smolka, 1995; Kraut, 1997) and human kidney (Kraut, 2001).

H,K-ATPase is present in the late distal tubule, collecting duct (Wingo and Smolka, 1995) and in the intercalated cell of rat cortical collecting tubule and outer medullary collecting tubule by immunoreactivity (Ahn and Kone, 1995; Wingo et al., 1990). It has been proposed that H,K-ATPase may be a candidate for potassium reabsorption in the renal distal tubule because this electroneutral pump exchanges potassium againt hydrogen ion and thus would also account, at least in part, for proton secretion prevailing in the collecting tubule (Figure D) (Sachs et al., 1982; Schwalbe et al., 2002).

Potassium reabsorption and hydrogen ion secretion have been shown to link functionally which is supported by the fact that acidosis is accompanied with alteration of luminal membrane of intercalated cell (Hagege et al., 1974). The similar result was also observed during potassium-depletion (Sachs et al., 1982). Both HK-α1 and HK-α2 are expressed in the collecting ducts of the kidneys of potassium-restricted rats or rabbits (Ahn et al., 1996b; Kraut et al., 1995).
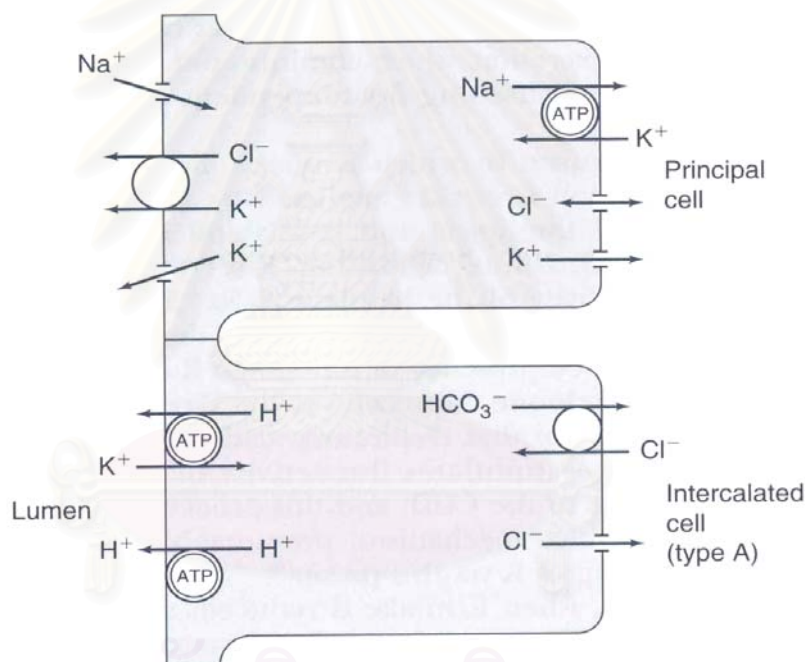
Figure D   Mechanism of K transport in the principal cell and intercalated cell of the cortical collecting duct (Schwalbe et al., 2002).

Effect of Vanadate on H,K-ATPase

H,K-ATPase is present in the intercalated cells of the collecting tubules and in gastric parietal cells. The pump drive protons in an electroneutral pattern. The H,K-ATPase is inhibited by vanadate and omeprazole (Defnis et al., 1992; Doucet and Marsy, 1987; Eiam-Ong et al., 1995a).

Distal renal tubular acidosis is common among Thai citizen in the Northeastern part of Thailand, representing one of the national health problems. Northeastern Thailand is the area known to have high vanadium content. Vanadium was also present in the urine of 30% of the people studied. There is a tendency to the development of potassium depletion during heat exposure in summer, inhibition of H,K-ATPase in the cortical collecting tubule by vanadium is a possibility (Sitprija et al., 1990).

Proton transport in the collecting tubule is directly mediated by a hydrogen-adenosine triphosphatase (H-ATPase) and hydrogen-potassium-adenosine triphosphatase (H,K-ATPase) and, is indirectly enhanced by a sodium-potassium- adenosine triphosphatase (Na,K-ATPase) (Alpern et al., 2000). It has been demonstrated that vanadate diminishs both H,K-ATPase and Na,K-ATPase activity in collecting tubules (Defnis et al., 1992). Therefore, vanadate could impair urinary acidification.

During acute metabolic acidosis, the kidneys secrete more hydrogen ions via the collecting tubule. This augments ammonium and titratable acid formation (Alpern et al., 2000). It has been shown that vanadate impairs the expected increase in urinary net acid excretion in acute acid-loaded animals (Eiam-Ong et al., 1996). The impairment of urinary acidification induced by vanadate may relate to its inhibitory effect on H,K-ATPase and

Na,K-ATPase. To date, there are no studies the effect of vanadate on either α- or β- isoform expression of these two enzymes.

Potassium Homeostasis

Potassium is the most abundant exchangeable cation in the body. Its distribution has previously been reported by Williams et al. (Williams and Epstein, 1989). In a 70 kg man, the body contains 3,500 mEq of potassium. Approximately 98% of potassium is confined to intracellular fluid at a concentration of about 150 mEq/liter. Seventy-seven percents of potassium distributes into muscle whereas 23% in other organs such as liver, bone, and red blood cell, etc. (Schwalbe et al., 2002; Williams and Epstein, 1989). The remaining 2% of the total body potassium is located in the extracellular fluid of about 14 liters. Normally, plasma K concentration is 3.5 to 5.5 mEq/liter (Guyton and Hall, 2000; Schwalbe et al., 2002). The concentration of potassium in the extracellular fluid is a critical determinant of neuromuscular excitability including cardiac, skeletal, and smooth muscle cells (Guyton and Hall, 2000). Small shifts of potassium into or out of cells may produce large changes in the plasma concentration (Schwalbe et al., 2002; Sterns et al., 1981). Therefore, the regulation of the extracellular potassium concentration has a considerable clinical importance. The extracellular potassium concentration is a function of two variables: 1) external potassium balance between intake and excretion, and 2) internal potassium balance between extra- and intracellular fluid.

1.1 External potassium balance

The average daily dietary intake of potassium is approximately 75 to 100 mEq. It is absorbed in the small intestine. About 90 to 95% of dietary potassium is excreted in urine each day, 5 to 10% in stool and less than 5% in sweat (Brown, 1984). Variations in potassium intake are matched within hours to day by parallel adjustments in potassium excretion through kidney (Berns and Hayslett, 1989; Brown, 1984).

1.2 Internal potassium balance

Internal potassium balance is determined by the distribution of potassium between the intracellular and extracellular fluids. The major biological mechanism maintaining the potassium gradient between intracellular fluid and extracellular fluid is the Na,K-ATPase dependent pump (Sterns et al., 1981). There are a variety of factors which affect the plasma potassium concentration by altering internal potassium balance such as the integrity of the cell membrane, plasma osmolality, acid-base status, and hormonal factors (Brown, 1984; Stanton and Koeppen, 1992; Sterns et al., 1981; Williams and Epstein, 1989; Wong, Schafer, and Schultz, 1993).

Renal Handling of Potassium

Potassium is freely filtered at the glomerulus and reabsorbed to a large extent along the proximal convoluted tubule (about 70%, Figure E) (Ganong, 2003). An additional 25% is reabsorbed by the loop of the Henle, so only about 5% of filtered potassium reaches the earliest portion of the distal tubule. It is along the late distal tubule and collecting tubule that major adaptations in potassium transport occur, depending on the

prevailing physiologic condition. Under normal conditions, potassium is secreted into the late portion of the distal tubule (connecting tubule) and throughout the cortical portion of the collecting tubule. In the outer medullary collecting tubule, there is no potassium transport (except in potassium depletion), whereas the inner medullary collecting tubule is capable of both potassium secretion and reabsorption (Guyton and Hall, 2000).
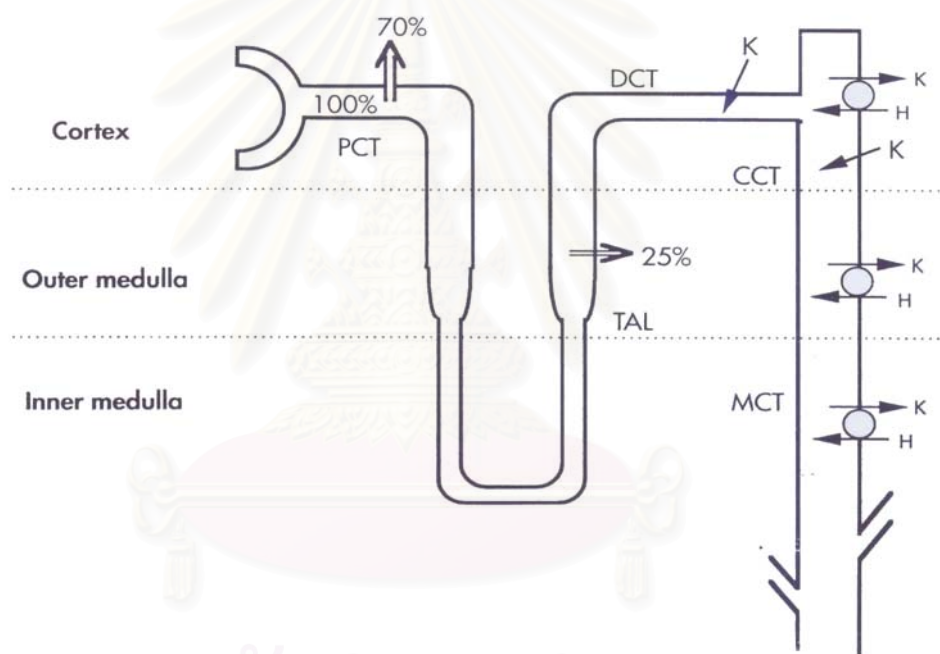
Figure E   Scheme of potassium handling in various nephron segments. As a result of potassium reabsorption in the proximal convoluted tubule (PCT) and thick ascending limb of Henle (TAL) only about 5% of the filtered potassium reaches the early distal convoluted tubule (DCT). Major changes in final urine potassium depend on potassium secretion in the late DCT and cortical collecting tubule (CCT). The collecting tubule may also be capable of potassium reabsorption by an H,K-ATPase. Arrows: direction of net potassium transport (Kaplan and Battle, 1995).

The appearance in the urine of more than 90% of all dietary potassium, ordinarily about 50 to 100 mEq per day, is the result of potassium secretion in the distal tubule and cortical collecting tubule (Schwalbe et al., 2002). Under conditions of dietary potassium excess, or hyperkalemia, potassium secretion in these nephron segments is greatly enhanced, whereas during potassium restriction, or hypokalemia, this secretory process is suppressed. Under the latter circumstances, there is potassium reabsorption within the collecting tubule, which further contributes to renal potassium conservation. The renal adaptation to excess potassium loads is fast near complete over a period of 24 to 72 hours, whereas the response to dietary deprivation is more sluggish and may require up to 1 week for full renal adaptation (Kaplan and Battle, 1995; Schwalbe et al., 2002; Stuart, 1999).

Hypokalemia

Hypokalemia is the state resulting from the disturbance of potassium homeostasis. It is usually defined as a serum potassium less than 3.5 mEq/liter (Schrock and Kuschinsky, 1989; Schwalbe et al., 2002). Clinically, mild hypokalemia is defined as a serum potassium ranging from 3.0 to 3.5 mEq/liter (Ganong, 2003; Schulman and Narins, 1990) whereas the level below 2.5 mEq/liter is considered as severe hypokalemia (Guyton and Hall, 2000; Ikram, 1987). However, serum potassium is not an accurate reflection of total body stores. With a similar serum potassium concentration, acute hypokalemia differs from that in chronic. The former result is a change in only the serum potassium concentration whereas the latter is accompanied by a reduction in both stores and serum levels (Schwalbe et al., 2002; Wong, Schafer and Schultz, 1993). Some studies

defined the severity of the K^+ depletion according to the depletion of skeletal muscle potassium content since it can be decreased to almost half of its regular content (Schrock and Kuschinsky, 1989). The etiologies of hypokalemia are due to many factors such as dietary insufficiency, gastrointestinal K^+ loss, renal K^+ loss, excessive sweat loss, or intracellular K^+ uptake alteration. Hypokalemia, by causing a compensatory exit of potassium ions across the basolateral membrane of renal cell, induces the reciprocal movement of H^+ into cell. The raised intracellular H^+ concentration leads to increase H^+ secretion and HCO_3^- reabsorption, causing a metabolic alkalosis. Another contributory factor may be stimulation (or increase insertion) of the luminal H,K-ATPase in α-intercalated cells (Unwin et al., 2002). Hypokalemia, also increase H,K-ATPase activity (Doucet and Marsy, 1987; Eiam-Ong et al., 1993; lbrahim et al., 1995) and Na,K-ATPase activity (Eiam-Ong et al., 1993) in outer medullary collecting tubule (Doucet and Marsy, 1987). In chronic hypokalemia enhances mRNA expression of the H,K-ATPase, both H,K-ATPase α1 in connecting segment and cortical collecting tubule (Ahn et al. 1996a) and H,K-ATPase α2 in the renal medulla (Ahn et al., 1996; Codina, Wall, and Dubose, 1999; Kraut et al., 1997).

CHAPTER III

MATERIALS AND METHODS

1. Experimental Animals

The study was performed in male Wistar rats, weighing 220 to 250 grams which were obtained from the National Center of Scientific Use of Animals (Mahidol University, Salaya, Nakhonpathom). The animals were housed in a well ventilated room in which the temperature was 23 to 25 °C with an automatic lighting schedule, which provided darkness from 8 p.m. to 6 a.m. The animals were given free access to standard laboratory chow and water as follow the experimental protocol. All rats were used once only and were kept in metabolic cages for 24 hours to collect urine before the experiment and before sacrificed. Urine volume was measured, and the samples were stored at - 80 °C until use.

2. Chemicals

2.1. Chemical Agents

Sodium pentobarbital (Nembutal®) was purchased from Sanofi, France. Absolute ethanol, 95% ethanol, Xylene, Dioxane, Di-sodium hydrogen phosphate (Na_2HPO_4), Charcoal, Normal HCl, Potassium di-hydrogen phosphate (KH_2PO_4), Trisma® acid, Trisma® base, Triton X-100, 30% hydrogen peroxide, and Gelatin were purchased from Merk, Germany. Paraformaldehyde, DAB (3, 3'-diamino-Benzidine), and sodium orthovanadate were purchased from Sigma, USA. Haematoxylin solution

(progressive stain) was purchased from C.V. Laboratories, Thailand. Soduim Bisulfite was purchased from AR®, USA. Paraffin pour embedding medium was purchased from St. Louis, USA. Low potassium diet (Catalog NO. 960189) was purchased from ICN Biomedicals, Ohio, USA.

2.2.　Antibodies

Mouse monoclonal antibody against Na,K-ATPaseα1 (Catalog NO. MA3-929) was purchased from ABR, USA. Mouse monoclonal antibody against H,K-ATPaseα1 (Catalog NO. D032-3) was purchased from MBL, Japan. Mouse monoclonal antibody against H,K-ATPaseα2 was generously provided by Dr. A Smolka, Univ. of South Carolina, Charleston, SC. Normal horse serum (Cat No. 16050-122, Lot. 434008) was purchased from GIBCO, New Zealand. Visualization reagent anti-mouse-rabbit immunoglobulin (DAKO EnVision™, Code No. K1491, Lot. 034246) was purchased from Dako, Denmark.

3.　Experimental Procedure

3.1.　Experimental Protocols

After three days to familiar with the new housing, the animals were weighed and collected blood samples from the tail for measuring BUN in order to assess kidney function (less than 30 mg %). The male Wistar rats were divided into 4 groups of eight each as follow and summerized in Table A:

1.   The rats were given normal saline solution; NSS (0.5 ml/kg BW/day) by intraperitoneal (i.p.) injection. These rats were received normal potassium diet; NK (K=150 mEq/kg diet) for 10 days.

2. The rats were given normal saline solution; NSS (0.5 ml/kg BW/day) by intraperitoneal (i.p.) injection. These rats were received low potassium diet; LK (K=1.5 mEq/kg diet) for 10 days.

3. The rats were given sodium orthovanadate; V (5mg/kg BW/day) by intraperitoneal (i.p.) injection. These rats were received normal potassium diet; NK (K=150 mEq/kg diet) for 10 days.

4. The rats were given sodium orthovanadate; V (5mg/kg BW/day) by intraperitoneal (i.p.) injection. These rats were received low potassium diet; LK (K=1.5 mEq/kg diet) for 10 days.
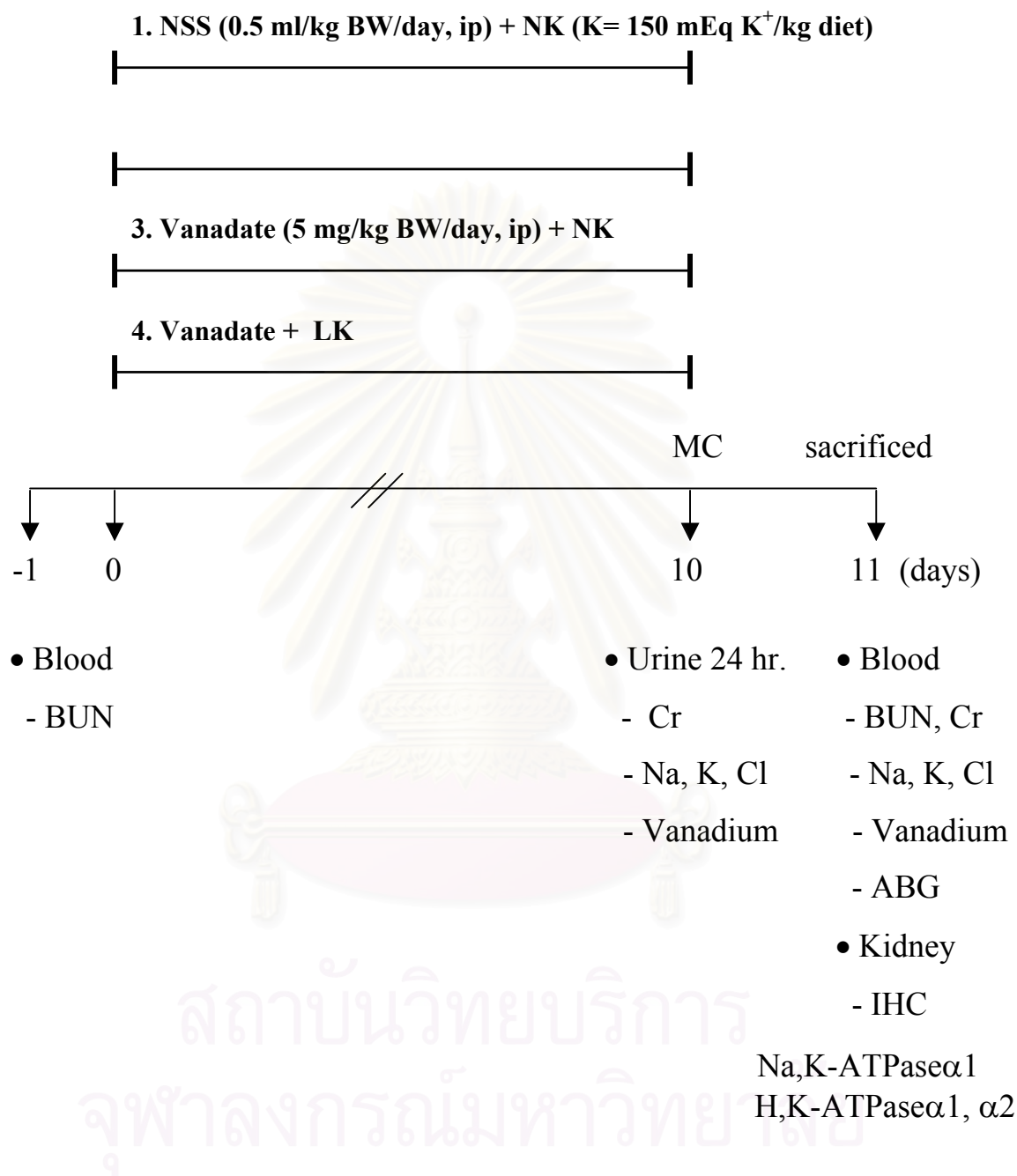
Table A. Experimental groups

Group	Treatment	
	i.p. injection	diet
1	NSS	NK
2	NSS	LK
3	V	NK
4	V	LK

n = 8 in each group; NSS = normal saline solution, V = sodium orthovanadate, NK = normal potassium diet, and LK = low potassium diet.

All animals were freed to access distilled water through the experimental period. Body weights were recorded at the beginning and at the end of the 10-day period. On the day before the experiment, the animals were placed in the metabolic cage for collection of twenty-four hour urine. On the due date, they were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (60 mg/kg BW), the abdomen was opened via a midline incision. Blood sample was collected from the abdominal aorta for measurement of arterial blood gas and then centrifuged at 3,500 rpm for 15 minutes. Serum was stored at -80°C until use for BUN, Cr, electrolyte, and vanadium measurements.

The kidneys were removed, the tissue sample was fixed in 10% paraformaldehyde overnight and embedded in paraffin for immunohistochemical detection of Na,K-ATPaseα1, H,K-ATPaseα1, and H,K-ATPaseα2 protein expression. In addition, the renal tissue samples (~200 mg wet wt) were initially dried heat for 24 hr and then measured for vanadium levels by atomic absorption spectrophotometer.

3.2. Experimental Design

1. NSS (0.5 ml/kg BW/day, ip) + NK (K= 150 mEq K$^+$/kg diet)

├───┤

├───┤

3. Vanadate (5 mg/kg BW/day, ip) + NK

├───┤

4. Vanadate + LK

├───┤

MC   sacrificed

-1   0   10   11 (days)

- Blood   • Urine 24 hr.   • Blood
  - BUN   - Cr   - BUN, Cr
                                   - Na, K, Cl   - Na, K, Cl
                                   - Vanadium   - Vanadium
                                                        - ABG
                                                      • Kidney
                                                        - IHC

                                              Na,K-ATPaseα1
                                              H,K-ATPaseα1, α2

MC	=	Metabolic cage
NK	=	Normal K diet (K= 150 mEq K$^+$/kg diet)
LK	=	Low K diet (K= 1.5 mEq K$^+$/kg diet)
ABG	=	Arterial blood gas
IHC	=	Immunohistochemistry

3.3. Immunohistochemical Study

Tissue sepecimens were fixed in buffer of 4% formaldehyde, dehydrated in ethanol, and then embedded in paraffin. Two-micromiter sections were cut by a microtome (Leica RM 2125 RT, Leica, USA.) and placed on glass sildes (SuperFrostPlus, Menzel, Germany). The sections were deparaffinized in xylene 3 times for 5 minutes each and absolute ethyl alcohol 3 times for 5 minutes each. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide (3% H_2O_2 in distilled water 2000 μL) for 10 minutes. The sections were then washed 2 times in PBS, pH 7.4 for 5 minutes each. The non-specific binding of the antibody was blocked by incubating tissue sections with 3% normal horse serum for 20 minutes at room temperature, and then drained the excess. The sections were then incubated in primary antibody for Na,K-ATPaseα1, H,K-ATPaseα1, or H,K-ATPaseα2 in concentrations of 1:2000, 1:10, and 1:500, respectively (diluted in 3% normal horse serum), for over one hour at room temperature.

After incubation, the sections were rinsed 2 times for 5 minutes each with PBS, pH 7.4 and were then incubated with visualization reagent anti-mouse-rabbit immunoglobulin (DAKO EnVision™, Denmark) for 30 minutes at room temperature. After incubation, the tissue sections were rinsed 2 times for 5 minutes with PBS, pH 7.4. Then, the sections were reacted for peroxidative activity by working DAB Tris-HCl buffer, pH 7.4 (3, 3'-diaminobenzidine tetrahydrochloride) for 10 minutes at room temperature. The sections were washed 5 minutes with distilled water. Finally, the slides were counterstained with haematoxylin and dehydrated. The sections were mounted and coverslipped with permount. Areas of staining were identified, and the intensity of staining was scored from 0 to 3

(0 = no staining, 1 = weak positive, 2 = moderate staining, and 3 = strongly positive staining) (Hegarty et al., 2001). All slides were viewed and scored by three blinded pathologists.

3.4. Vanadium Measurement

Kidney, urine, and plasma samples from all groups were analyzed for vanadium concentration by using electrothermal atomic absorption spectrophotometer (Model 4110ZL AA spectrometer, Perkin-Elmer Corp., Ueberlingen, Federal Republic of Germany).

3.5. Electrolyte Measurement

Plasma and urine samples from all groups were analyzed for Na, K, Cl, HCO_3, BUN, Cr by using ISE (ion selection electrode) indirect method (Model CX3, Beckman Instruments INC, Germany).

3.6. Arterial Blood Gas Analysis

Blood samples were obtained from abdominal aorta and immediately measured for arterial blood gas (ABG) by a blood gas analyzer (Osmetech OPTI™ CCA; Model NO. OPTI3, Serial NO. OP3-4379) Roche, USA.

3.7. Calculation for Assessment of Renal Function

$$\text{Creatinine clearance } (C_{Cr}) = \frac{U_{Cr} \times V}{P_{Cr}}$$

$$\text{Urinary electrolyte excretion} = U_e \times V$$

$$\text{Fractional electrolyte excretion } (FE_e) = \frac{U_e V/P_e \times 100}{C_{Cr}}$$

4. Statistical Analysis

All data were expressed as mean $\pm$ S.E. The results of blood and urine parameters were compared by using ANOVA and followed by Duncan analysis. Probability values of less than 0.05 were considered to be statistically significant. The intensity scores of Na,K-ATPaseα1, H,K-ATPaseα1, and H,K-ATPaseα2 protein expression were presented in descriptive statistics by measures of central tendency (Mode).

CHAPTER IV

RESULTS

Vanadium Levels in Serum, Urine, and Renal Tissues

The concentrations of vanadium in serum, urine, and renal tissues are shown in Table 1. Ten days of vanadate (V) administration significantly increased vanadium levels in serum, urine, and renal tissues in both normal-potassium (NK) and low-potassium (LK) diet groups.

Interestingly, the LK-diet rats treated with vanadate had a lesser vanadium concentration in serum and urine as compared with the respective NK-diet group (558.38 ± 24.98 vs 678.15 ± 44.62 ng/ml, and 711.03 ± 17.5 vs $2,658.20 \pm 78.15$ ng/ml, respectively; $p < 0.001$). By contrast, in V + LK group, the vanadium accumulation was demonstrated in a greater extent in both renal cortex and medulla tissues as compared with those of V + NK animals ($p < 0.001$). Of noted, vanadium was deposited in cortical region approximately double when compared to those in medulla area ($p < 0.001$).

Metabolic Parameters in Rats Treated with Vanadate or/and Low Potassium Diet

As shown in Table 2, low potassium diet for ten days markedly caused hypokalemia in both normal saline solution (NSS) and vanadate treated animals ($p < 0.001$).

In normal K-diet group, ten days of vanadate injection significantly decreased serum potassium (from 3.96 ± 0.12 to be 3.25 ± 0.13 mmol/L;

$p < 0.001$) as well as plasma chloride (from 106.75 ± 0.49 to be 101.63 ± 0.60 mmol/L; $p < 0.05$).

In low K-diet rats, after vanadate administration, both serum potassium and chloride were progressively declined to be 1.63 ± 0.10 and 87.13 ± 2.64 mmol/L, respectively; $p < 0.001$. Moreover, serum sodium also was reduced significantly in LK-diet animals treated with vanadate. Neither serum creatinine nor blood urea nitrogen was altered significantly by low K-diet alone. However, the combination treatment with vanadate caused azotemia. Serum creatinine was increased almost 3 times ($p < 0.001$) whereas blood urea nitrogen was enhanced by 4 times ($p < 0.001$) when compared to the other groups (NSS + NK, NSS + LK, and V + NK groups).

No significant changes in serum bicarbonate of both normal saline solution and vanadate treated groups (to be 29.63 ± 0.54 and 28.13 ± 1.09 mmol/L, respectively).

Interestingly, in low K-diet animals, blood pCO_2 levels were slightly declined ($p < 0.05$) as compared with their respective normal saline solution treated groups. Vanadate administration in normal K-diet rats trended to increase blood pCO_2 but it did not reach significance. All animals studied were in normal levels of blood pH and blood pO_2 (Table 2).

Table 1   Vanadium levels in rats treated with normal potassium diet or
low potassium diet (Mean $\pm$ SEM, n = 8/group)

Groups Parameters	NSS		V	
	NK	LK	NK	LK
Serum (ng/ml)	0.25 $\pm$ 0.01	0.15 $\pm$ 0.02	678.15**, ♠♠ $\pm$ 44.62	558.38**, ♠♠,# $\pm$24.98
Urine (ng/ml)	0.38 $\pm$ 0.05	0.42 $\pm$ 0.04	2,658.20**, ♠♠ $\pm$ 78.15	711.03**, ♠♠,# $\pm$17.53
Renal Cortex (μg/g. dry wt.)	0.20 $\pm$ 0.04	0.18 $\pm$ 0.04	34.98**, ♠♠ $\pm$ 0.84	79.90**, ♠♠,# $\pm$ 6.06
Renal Medulla (μg/g. dry wt.)	0.16 $\pm$ 0.06	0.11 $\pm$ 0.03	17.73**, ♠♠ $\pm$ 3.66	44.90**, ♠♠,# $\pm$ 4.64

NSS = normal saline solution   NK = normal potassium diet   ** $p < 0.001$ vs NSS + NK
V   = vanadate   LK = low potassium diet   ♠♠ $p < 0.001$ vs NSS + LK
                                                              # $p < 0.001$ vs V + NK

**Table 2   Metabolic parameters in rats treated with vanadate or/and
low   potassium diet (Mean $\pm$ SEM, n = 8/group)**

Groups Parameters	NSS		V	
	NK	**LK**	**NK**	**LK**
S_{Na+} (mmol/L)	140.75 $\pm$ 0.41	142.00 $\pm$ 0.57	143.88 $\pm$ 0.69	138.88*, ♠, + $\pm$ 1.48
S_{K+} (mmol/L)	3.96 $\pm$ 0.12	1.71** $\pm$ 0.04	3.25* $\pm$ 0.13	1.63**, # $\pm$ 0.10
S_{Cl-} (mmol/L)	106.75 $\pm$ 0.49	105.38 $\pm$ 0.71	101.63* $\pm$ 0.60	87.13*, ♠♠,# $\pm$ 2.64
S_{HCO3-} (mmol/L)	27.65 $\pm$ 0.76	26.60 $\pm$ 0.81	29.63 $\pm$ 0.54	28.13 $\pm$ 1.09
Blood pH	7.45 $\pm$ 0.01	7.51 $\pm$ 0.02	7.42 $\pm$ 0.02	7.53 $\pm$ 0.03
Blood pCO$_2$ (mmHg)	41.25 $\pm$ 2.02	34.38* $\pm$ 2.34	47.13 $\pm$ 2.75	34.50 $\pm$ 2.30
Blood pO$_2$ (mmHg)	104.00 $\pm$ 3.74	117.63 $\pm$ 4.47	91.38 $\pm$ 6.94	107.00*, + $\pm$ 4.16
S_{Cr} (mg/dL)	0.34 $\pm$ 0.02	0.51 $\pm$ 0.04	0.42 $\pm$ 0.02	1.50**, ♠♠,# $\pm$ 0.22
BUN (mg/dL)	16.75 $\pm$ 0.67	18.63 $\pm$ 1.10	15.50 $\pm$ 1.49	83.38**, ♠♠,# $\pm$ 2.06

NK = normal potassium diet, LK = low potassium diet, NSS = normal saline solution,
V = vanadate, S = serum, Cr = Creatinine, BUN = blood urea nitrogen

* $p < 0.05$ vs NSS + NK,   ♠ $p < 0.05$ vs NSS + LK,   + $p < 0.05$ vs V + NK,

**$p < 0.001$ vs NSS + NK,   ♠♠ $p < 0.001$ vs NSS + LK,   # $p < 0.001$ vs V + NK

Renal Function in Rats Treated with Vanadate or/and Low Potassium Diet

As shown in Table 3, ten days of vanadate treatment in normal-K diet rats did not affect renal function. Low K-diet with normal saline solution treatment significantly decreased fractional excretion of potassium (FE_K) from 63.24 ± 3.50 to be 10.08 ± 0.93 % ($p < 0.001$, Figure 1) as well as had a decline in creatinine clearance (C_{cr}) by 50% (from 0.91 ± 0.08 to be 0.47 ± 0.04 ml/min/100 g BW; $p < 0.05$). This value was progressively reduced to be 0.13 ± 0.03 ml/min/100g BW ($p < 0.001$) when the animals received both vanadate and low K-diet.

Although the rats were received low K-diet, vanadate treatment increased FE_K to be 307.18 ± 9.97 % ($p < 0.001$). The significantly higher levels of FE_{Cl} and FE_{HCO3} were also observed in these animals by 3 times ($p < 0.05$) and 7 times ($p < 0.001$), respectively. Furthermore, the low K-diet rats exposed to vanadate for ten days had natriuresis. The values of FE_{Na} were approximately double as compared with the other groups (NSS + NK, NSS + LK, and V + NK groups). All rats in the experiment showed no significant changes in urine flow rate (Table 3).

Table 3   Renal function in rats treated with vanadate or/and low potassium diet (Mean $\pm$ SEM, n = 8/group)

Groups Parameters	NSS		V	
	NK	LK	NK	LK
C_{cr} (ml/min/100g BW)	0.91 $\pm$ 0.08	0.47* $\pm$ 0.04	0.89 $\pm$ 0.09	0.13**, ♠♠,# $\pm$ 0.03
FE_{Na^+} (%)	0.72 $\pm$ 0.01	0.61 $\pm$ 0.10	0.66 $\pm$ 0.04	1.53*, ♠, + $\pm$ 0.53
FE_{K^+} (%)	63.24 $\pm$ 3.50	10.08** $\pm$ 0.93	70.56 $\pm$ 7.90	307.18**, ♠♠,# $\pm$ 9.97
FE_{Cl^-} (%)	1.37 $\pm$ 0.11	0.88 $\pm$ 0.03	1.14 $\pm$ 0.09	3.36*, ♠, + $\pm$ 0.79
$FE_{HCO_3^-}$ (%)	3.82 $\pm$ 0.77	2.53 $\pm$ 0.45	3.02 $\pm$ 1.06	21.80**, ♠♠,# $\pm$ 1.87
V (ml/day/100g BW)	13.99 $\pm$ 1.43	15.90 $\pm$ 1.37	10.29 $\pm$ 0.98	11.04 $\pm$ 1.77

NK = normal potassium diet, LK = low potassium diet, NSS = normal saline solution, V = vanadate, C_{Cr} = Creatinine clearance, BW = body weight, FE = fractional excretion, V = urine flow rate

* p< 0.05 vs NSS + NK, ♠ p< 0.05   vs NSS + LK, + p< 0.05 vs V + NK

** p< 0.001 vs NSS + NK, ♠♠ p< 0.001 vs NSS + LK, # p< 0.001 vs V + NK
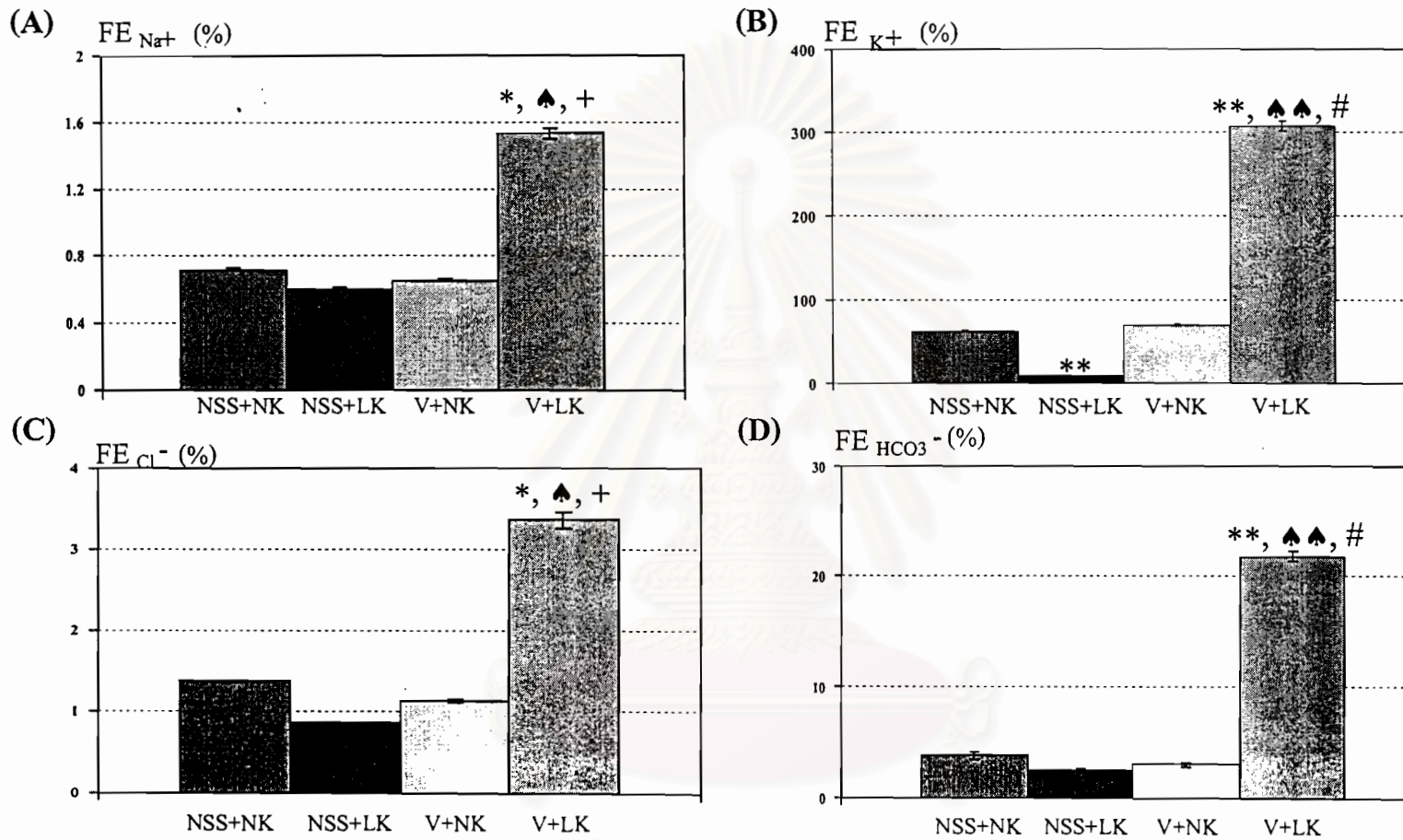
Figure1 Fractional excretion of Na^{+}, K^{+}, Cl^{-}, and HCO$_3^-$ in rats with vanadate or/and low K-diet rats (n=8/ group). NSS= normal saline solution, V = vanadate, NK =normal potassium diet, LK =low potassium diet.

    *   p<0.05   vs NSS + NK,   ♠   p<0.05   vs NSS + LK,   +   p<0.05   vs V + NK

    ** p<0.001 vs NSS + NK,   ♠♠ p<0.001 vs NSS + LK,   ++ p<0.001 vs V + NK
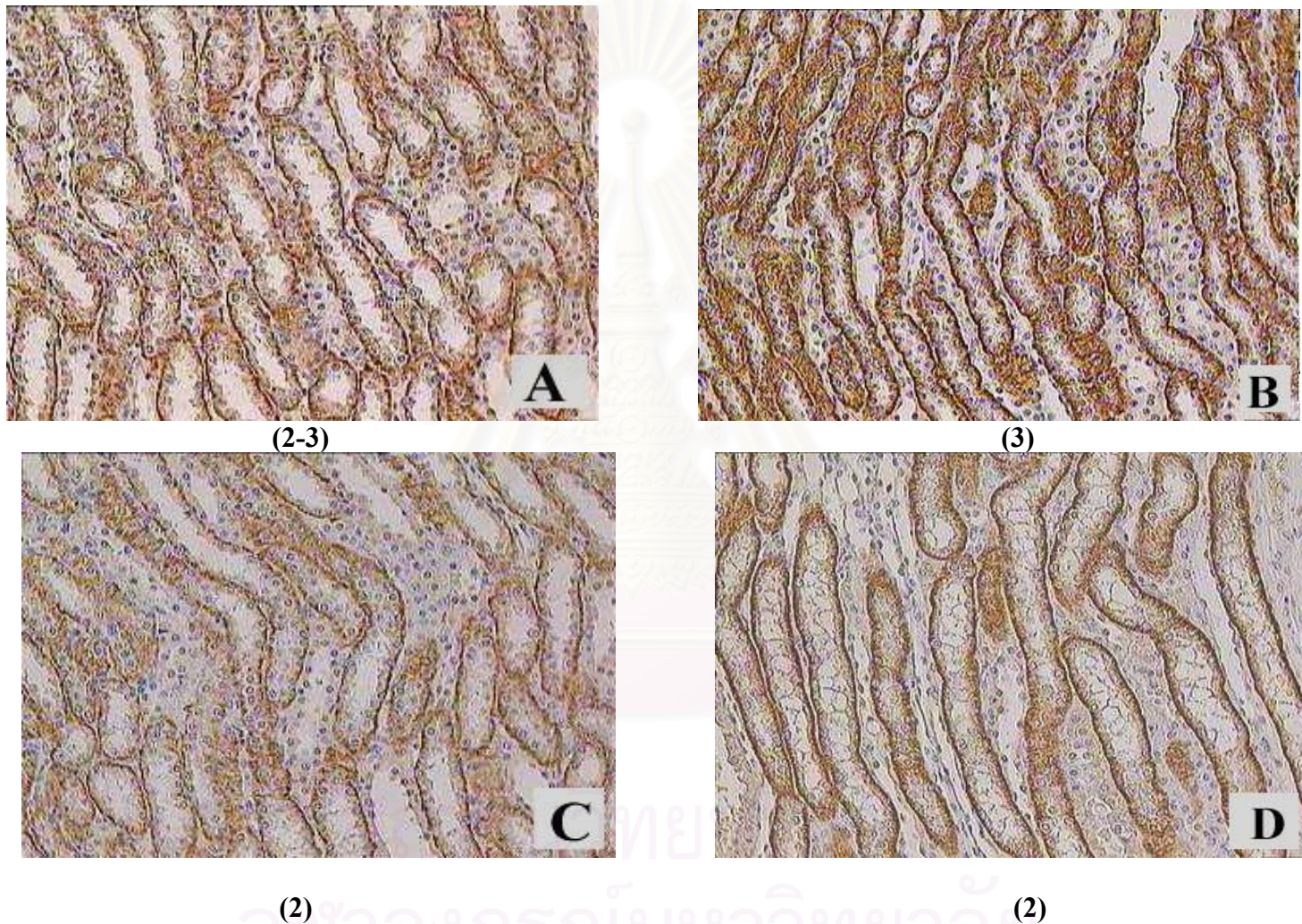
Renal Na,K-ATPase Protein Expression

The expression of Na,K-ATPase protein in renal tissues detected by immunohistochemical method is shown in Figures 2 and 3. In all groups, the expression occurred in tubular epithelium, but not in glomeruli. The staining of Na,K-ATPase protein was presented in cortex as well as in medulla at basolateral membrane. The prominent staining areas were observed in distal tubule and collecting ducts whereas the pale expression was noted in proximal tubule regions.

Ten days of low K-diet administration, in normal saline solution treated animals, diffusively enhanced the protein expression both in cortex and medulla at basolateral membrane. In distal tubules and collecting ducts, the intensity scores were increased from 2 – 3 to be 3 (Figures 2B and 3B; Table 4). By contrast, ten days of vanadate injection in normal K-diet rats caused a slight decline in the protein expression in renal tissue regions. The intensity scores were lower to be 2 (Figure 2C and 3D, Table 4). Interestingly, potassium depletion could not restore the suppressive effect of vanadate. The intensity scores were still at 2 in both cortex and medulla (Figure 2D and 3D, Table 4).
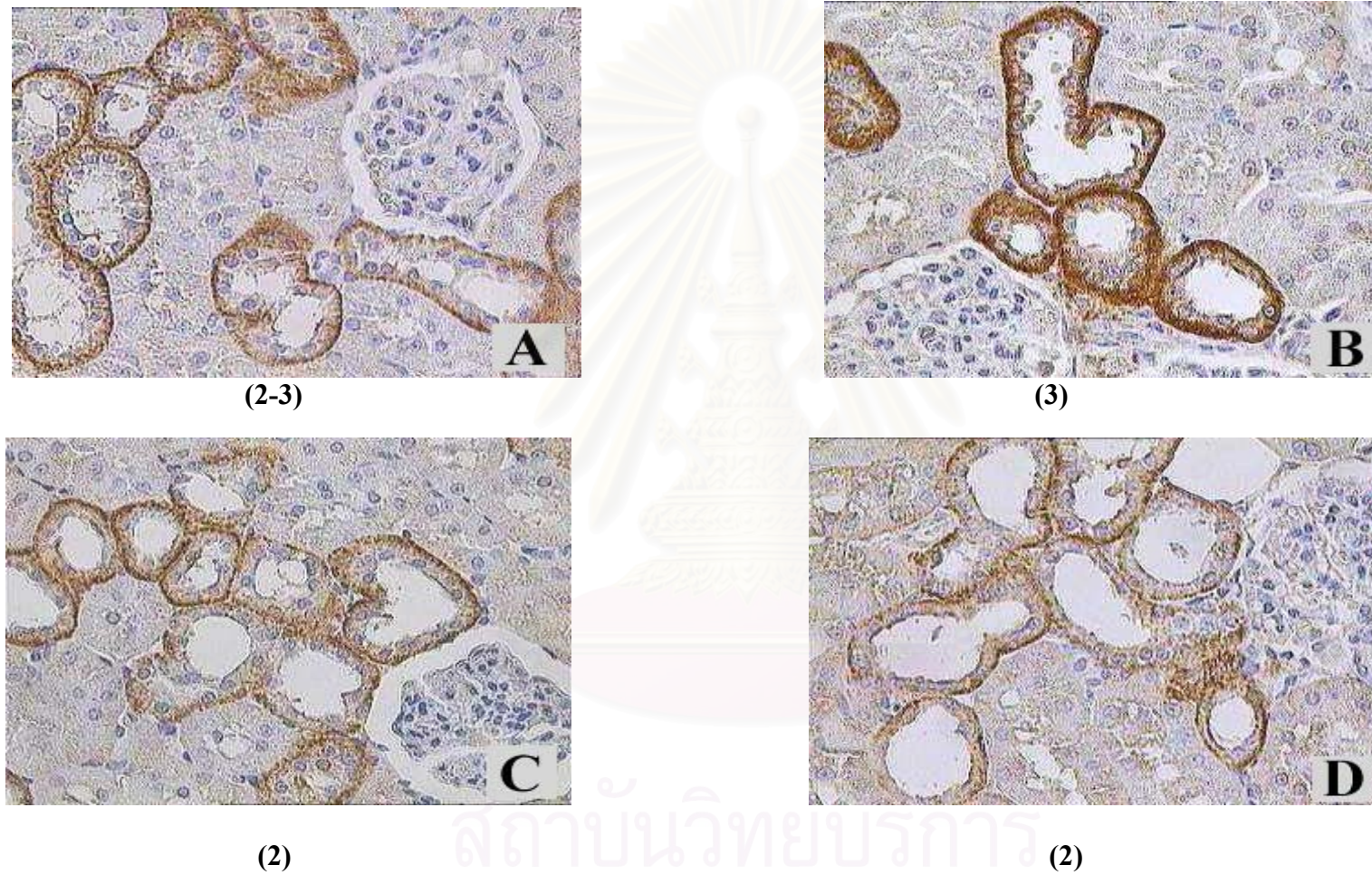
Figure 2 Immunohistochemical staining of renal cortex Na,K-ATPase protein expression. A: NSS+NK, B: NSS+LK, C: V+NK, and D: V+LK. Intensity scores are presented in parenthesis. Original magnification: 400X.

Figure 3 Immunohistochemical staining of renal medulla Na,K-ATPase protein expression. A: NSS+NK, B: NSS+LK, C: V+NK, and D: V+LK. Intensity scores are presented in parenthesis. Original magnification: 400X.

Table 4 The intensity scores of renal Na,K-ATPase, H,K-ATPaseα1, and H,K-ATPaseα2 protein expression in rats treated with vanadate or/and low potassium diet (Mode, n = 8/group)

Parameters	Groups	NSS		V	
		NK	LK	NK	LK
Na,K-ATPase	cortex	2-3	3	2	2
	medulla	2-3	3	2	2
H,K-ATPase (α1)	cortex	1	2	1	2
	medulla	0	1	0	1
H,K-ATPase (α2)	cortex	0-1	2	0-1	1
	medulla	0	0	0	0

NSS = normal saline solution, V = vanadate, NK = normal potassium diet, LK = low potassium diet.

The intensity of staining was scored from 0 to 3 (0 = no staining, 1 = weak positive, 2 = moderate staining, and 3 = strongly positive staining).

All slides were viewed and scored by three blinded pathologists.

Renal H,K-ATPaseα1 Protein Expression
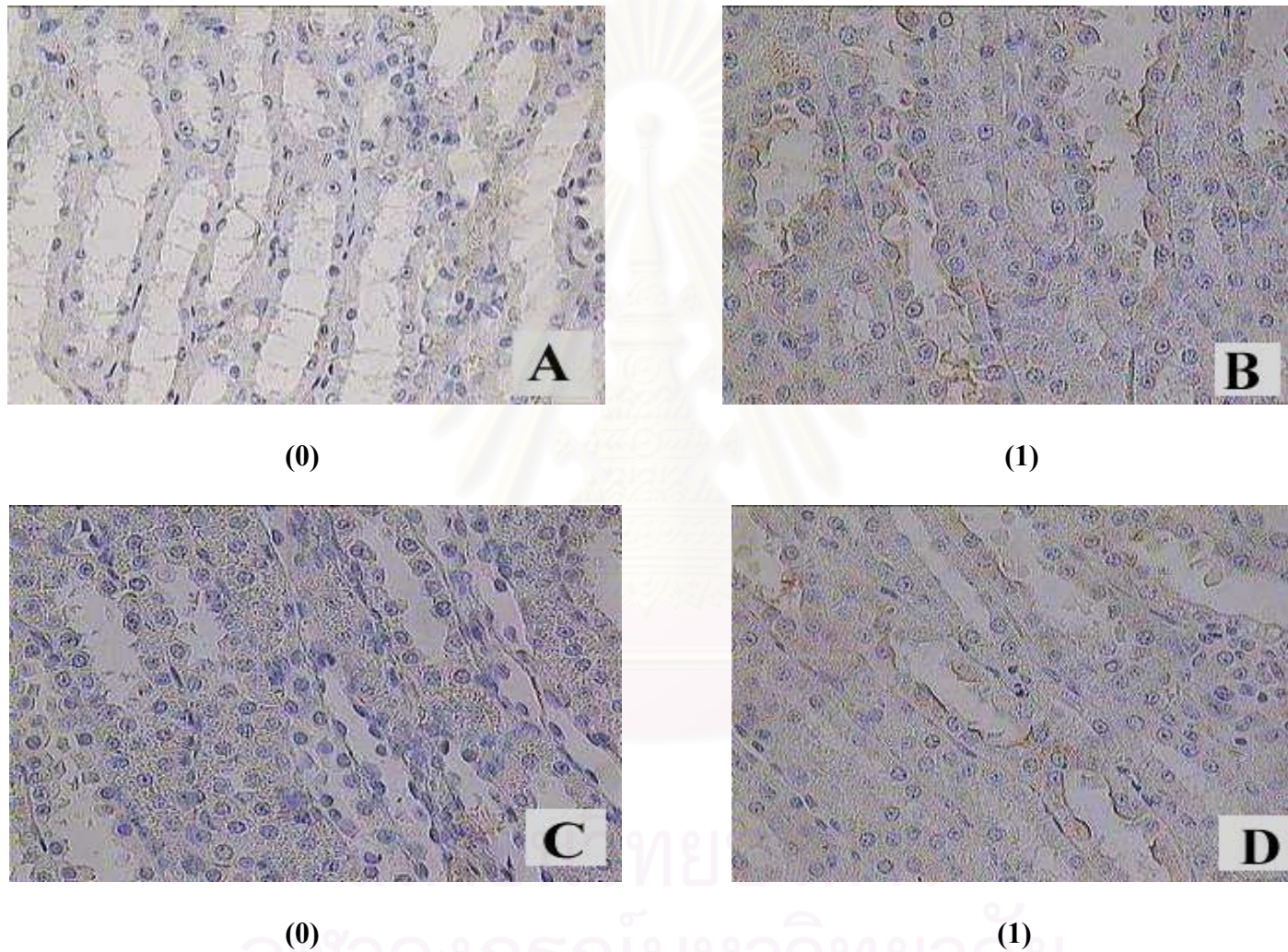
The expression of H,K-ATPaseα1 protein detected by immunohistochemical method is shown in Figures 4 and 5. Most staining was appeared at luminal membrane of collecting ducts. No staining was noted in glomeruli.

Ten days of vanadate injection had no effect on the protein expression both in cortex and medulla (Figure 4C and 5C, respectively; Table 4). However, low K-diet could enhance the expression from the scores of 1 to be 2 in cortical areas. These occurred in both normal saline solution (Figure 4B) and vanadate treated rats (Figure 4D). In medulla, the expression also presented in the same manner that low K-diet administration stimulated the expression by increasing the scores from 0 to be 1 (Figure 5B and 5D, Table 4).

Of noted, the more staining areas of H,K-ATPaseα1 protein were observed in cortex than those in medulla.
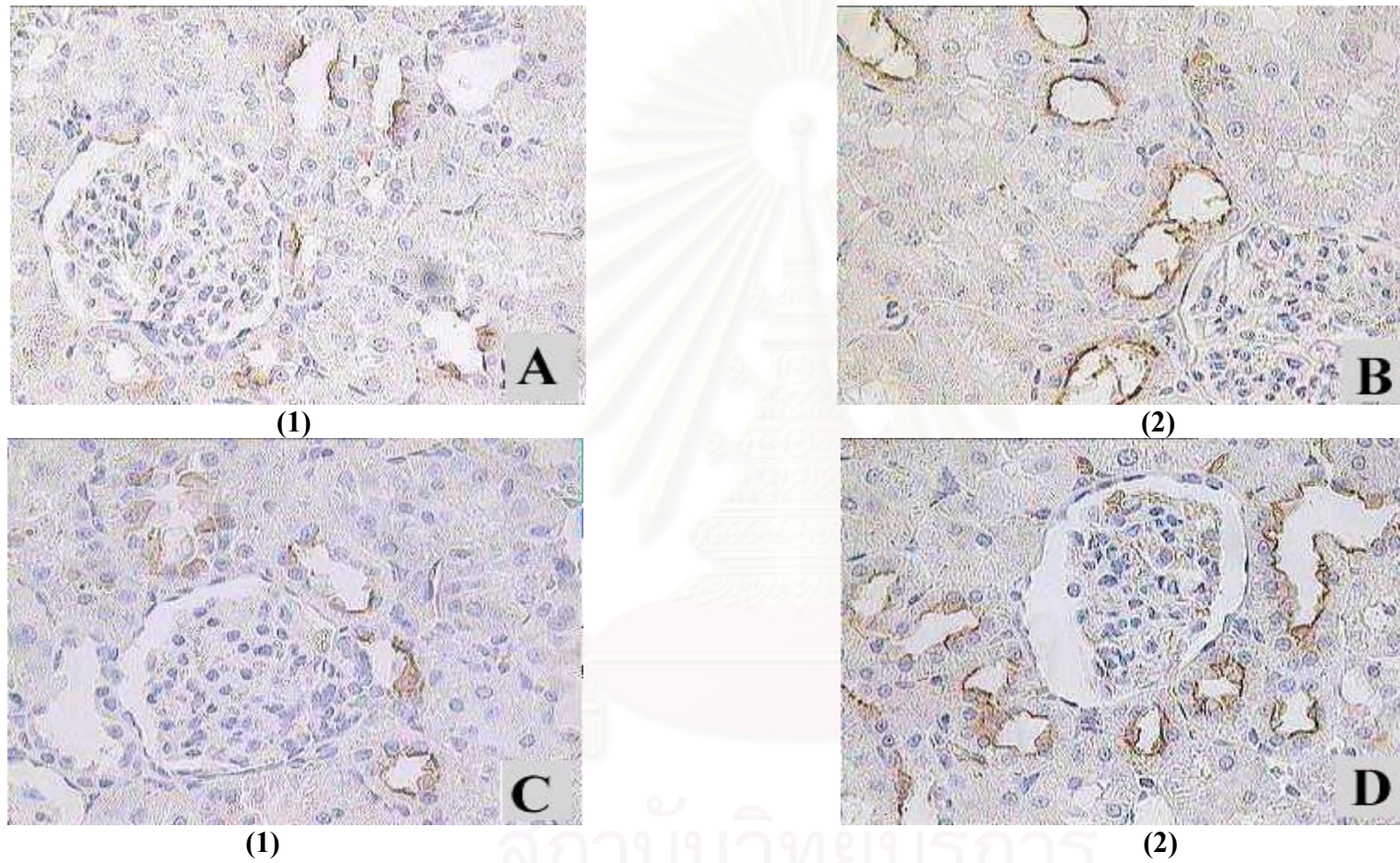
Figure 4   Immunohistochemical   staining of renal cortex H,K-ATPaseα1 protein expression.   A: NSS+NK, B: NSS+LK, C: V+NK, and D: V+LK. Intensity scores are presented in parenthesis. Original magnification: 400X.

Figure 5 Immunohistochemical staining of renal medulla H,K-ATPaseα1 protein expression. A: NSS+NK, B: NSS+LK, C: V+NK, and D: V+LK. Intensity scores are presented in parenthesis. Original magnification: 200X.

Renal H,K-ATPaseα2 Protein Expression

The expression of H,K-ATPaseα2 protein detected by immunohistochemistry is shown in Figures 6 and 7. Most staining was appeared at luminal membrane of cortical collecting ducts. No staining was noted in glomeruli.

Ten days of vanadate administration did not alter the protein expression in cortical regions (Figure 6C). However, low K-diet for ten days increased the cortical protein expression from the scores of 0-1 to be 2 in normal saline solution treated rats (Figure 6B, Table 4). In vanadate treated group, the expression showed a lesser extent by enhancing to be the score only 1 (Figure 6D, Table 4).

Interestingly, no expression of H,K-ATPaseα2 protein was detected in medulla areas from all groups studied (Figure 7, Table 4).

Figure 6 Immunohistochemical staining of renal cortex H,K-ATPaseα2 protein expression. A: NSS+NK, B: NSS+LK, C: V+NK, and D: V+LK. Intensity scores are presented in parenthesis. Original magnification: 400X
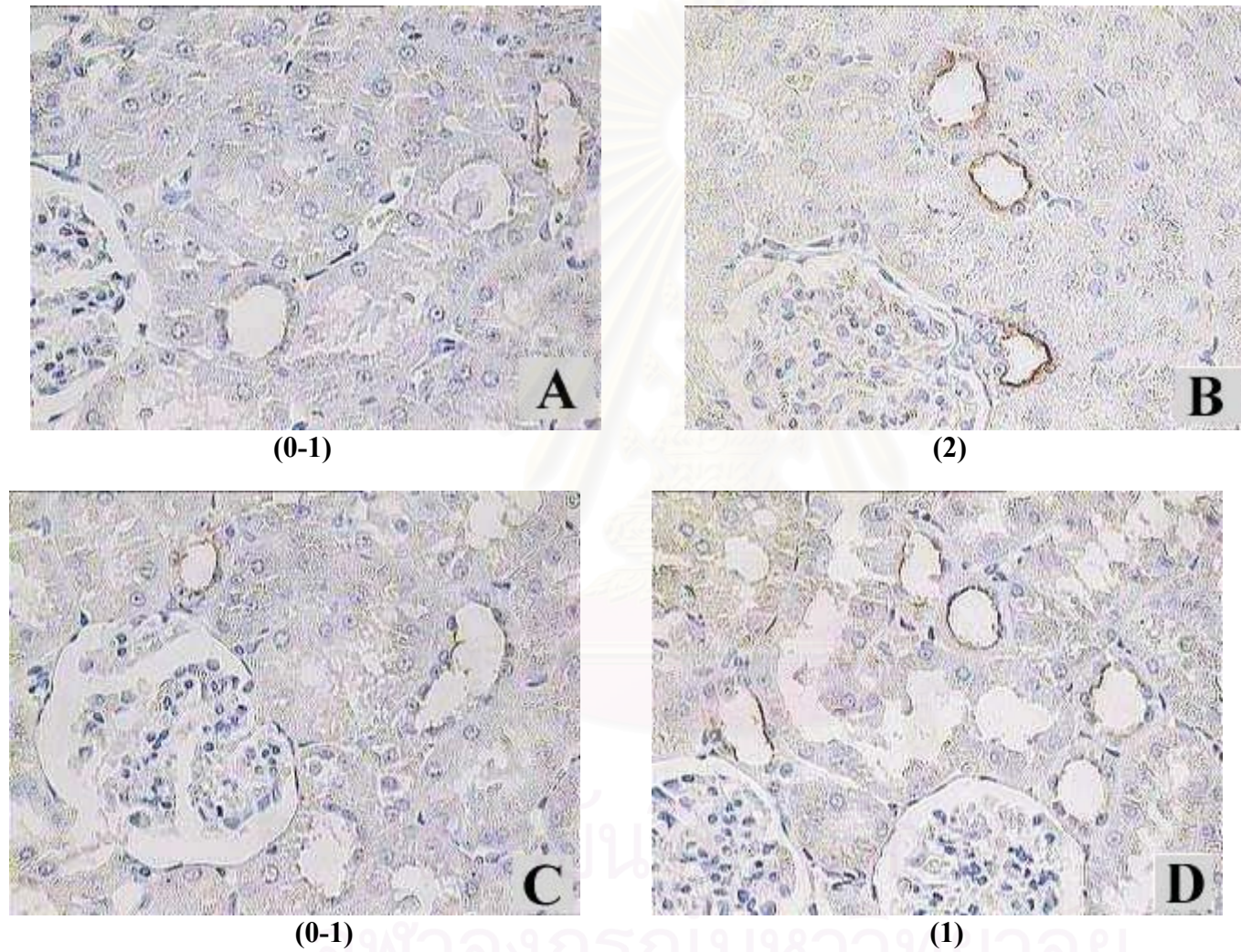
(0)

(0)

(0)

(0)
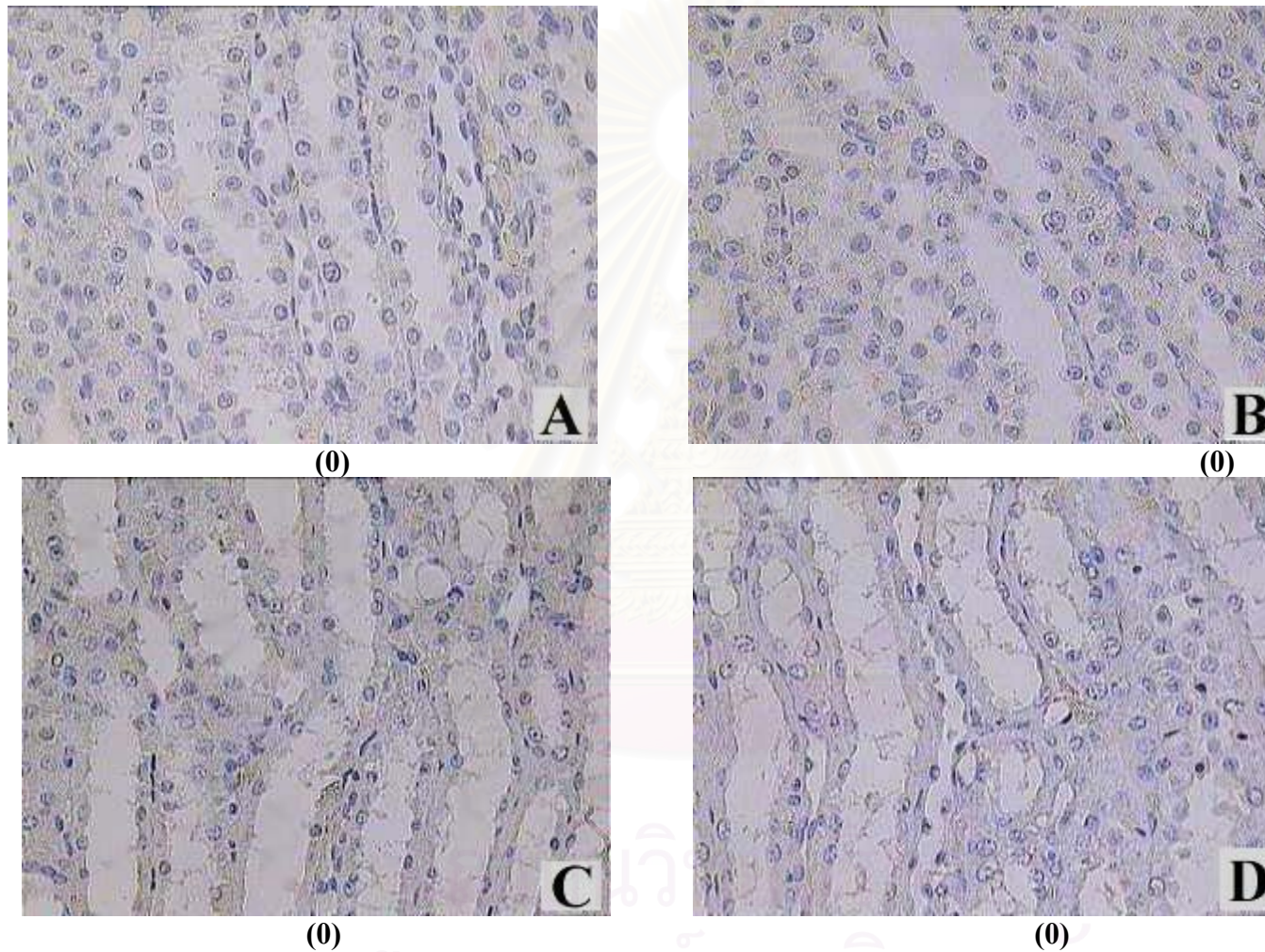
Figure 7 Immunohistochemical staining of renal medulla H,K-ATPaseα2 protein expression. A: NSS+NK, B: NSS+LK, C: V+NK, and D: V+LK. Intensity scores are presented in parenthesis. Original magnification: 200X

CHAPTER V

DISCUSSION AND CONCLUSION

It has been demonstrated that vanadium could affect on renal hemodynamics and tubular function (Dafnis and Sabatini, 1994). Vanadate administration has been shown to suppress the activity of Na,K-ATPase in collecting duct (Dafnis et al., 1992), purified or microsomal preparations of renal tissues (Clough, 1985; Neider et al., 1979; Nechay and Saunders, 1978; Grantham and Glynn, 1979), renal tissue sections (Boscolo et al., 1994), red blood cell (Cantley et al., 1978a; Karlish et al., 1979), skeleton muscle (Cantley et al., 1977), and cardiac muscle (Clough, 1985). The inhibition effect of vanadate on Na,K-ATPase activity has been clearly revealed that vanadate blocks a conformational change of the unphosphorylated form of Na,K-ATPase at the cytoplasmic side (Grantham, 1980; Phillips et al., 1983).

Despite many studies on Na,K-ATPase activity of vanadate, its protein expression was not revealed. In the present study, ten days of intraperitoneal injection of vanadate significantly increased vanadium levels in plasma, urine, and renal tissues. Of noted, vanadium was deposited in cortical region approximately double when compared to those in medulla area (Table 1). By immunohistochemistry the present finding is the first document showing that vanadate suppresses the protein expression of Na,K-ATPase (Table 4). The precised mechanism of this alteration remains unknown. The explanation may involve in various cellular regulatory cascades of vanadium.

Vanadium is a trace metal in the environment and in biological systems (Stern et al., 1993). The biologically relevant status of vanadium

are the following: vanadate V (V), the pentavalent state (usually an oxyanion, i.e., HVO_4^{2-} or H_2VO^{4-}); vanadyl V (IV), the tetravalent state (usually an oxycation, i.e., VO^{2+}); and vanadium (III). Vanadium compounds readily undergo redox reactions in presence of reductants, oxygen, or its reduced products (Stern et al., 1993). Vanadyl V (IV) spontaneously reacts with oxgen to yield vanadate V (V) and superoxide (Liochev and Fridovich, 1990). Vanadate V (V) and superoxide may then form a peroxyvanadyl complex that can oxidize NAD(P)H in a chain reaction (Stern et al., 1993).

Vanadyl has only limited ability to cross cell membranes as the free cation (Heinz et al., 1982), but vanadate enters the cell through nonspecific anion channels and is reduced intracelllularly to vanadyl (Cantley et al., 1978b; Heinz et al., 1982). Once in the cell, vanadyl is bound to protein sulfhydryl group of glutathione, and ascorbate (Macara et al., 1980; Nechay et al., 1986). Injected vanadium is rapidly removed from the blood plasma (where it is mainly carried on transferrin) and distributed to kidney, liver, testes, bone, and spleen (Chasteen et al., 1986). Vanadium is also found in nuclei and mitochondria (Oberg et al., 1978; Bracken et al., 1985). Therefore, vanadium could affect on cellular function and gene expression (Stern et al., 1993).

It has been shown that vanadate could modulate gene expression by activates certain transcription factors, such as AP-1 (Ding et al., 1999), NF-κB (Ye et al., 1999; Chong et al., 2000), p53 (Huang et al., 2000; Zhang et al., 2002), and nuclear factor of activated T cells (NFAT) (Huang et al., 2001). The AP-1 transcription factor consists of Jun/Jun homodimers or heterodimers of Jun (c-Jun, Jun B, and Jun D) and Fos (c-Fos, Fos B, Fra-1, and Fra-2) (Ding et al., 1999). Indeed, both c-*jun* and c-*fos* gene expression have been enhanced by vanadate (Wang and Scott, 1992; Wang

et al., 1997; Yin et al., 1992; Chen and Chan, 1993; Jin et al., 2000). Therefore, the reduction of Na,K-ATPase protein expression in the present result may be regulated, in part, through alteration of these transcriptional factors. Further studies in this regard as well as in mRNA expression, and protein level of Na,K-ATPase remain to be elucidated.

The present study also shows the first effect of vanadate on H,K-ATPase protein expression (Table 4). Surprisingly, vanadate had no influence on H,K-ATPase protein expression. Since vanadate could reduce this enzyme activity in renal tubule segments (Dafnis et al., 1992; Eiam-Ong et al., 1995b), and in intact gastric vesicle (Saccomani et al., 1977; Faller et al., 1983). Similar to Na,K-ATPase, vanadate could inhibit the H,K-ATPase by binding competitively with ATP at catalytic site (Faller et al., 1983). The failure to inhibit H,K-ATPase protein expression by vanadate despite suppressing its activity implies that the process of transcription or translation of H,K-ATPase is unaffected. More further investigations remain to be examined in these regards. However, the specificity of vanadate on selectively altered ATPase suggests that vanadate has a distinct mode of regulation and mechanism of action on these enzymes.

In addition, vanadate also can stimulate a variety of enzymes, including adenylate cyclase, glyceraldehyde-3-phosphate dehydrogenase, NADPH oxidase, tyrosine phosphorylase, glycogen synthase, lipoprotein lipase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, and cytochrome oxidase (Erdmann et al., 1984; Nechay et al., 1986). Furthermore, protein tyrosine kinase, MAP kinase, PKB, PKD, PKC, and PLC have also been activated by vanadate (Zor et al., 1993; Elberg et al., 1994; Natarajan et al., 2001; Molero et al., 2002; Torres and Forman, 2002; Luo et al., 2003; Li et al., 2004; Wenzel et al., 1995). By contrast, vanadate

inhibits acid and alkaline phosphatases, phosphodiesterase, phosphotyrosyl phosphatase, ribonuclease, Ca^{++}-ATPase, and UDP-*N*-acetylglucosaminyl transferase (Ramasarma and Crane, 1981; Swarup et al., 1982; Chasteen, 1983; Marshall and Okuyama, 2004).

Since vanadium could be found in nuclei, alterations in gene expression would occur. For instance, vanadate has activated gene expression of Glut-1 (Mounjoy and Flier, 1990), gene 33 (Weinstrock and Messina, 1992), PDGF B chain (Wenzel et al., 1995), class A scavenger receptor (Mietus-Synder et al., 1998), macrophage inflammatory protein-2 (Chong et al., 2000a), p21 (Zhang et al., 2001), glucocorticoid receptor (Calzi et al., 2002), ICAM-1 (Aduette et al., 2001), SOCS3 (Kita et al., 2003), and phosphatidylinosito-3 kinase (Zhang et al., 2004). On the other hand, vanadate could inhibit P-enolpyruvate carboxykinase gene expression (Bosch et al., 1990).

Regard to potassium administration, in the present study, LK diet alone for ten days increased Na,K-ATPase protein expression both in cortex and medulla. The protein staining still remained at basolateral membrane. This result is agreed with the study of Buffin-Meyer et al. (1998) performed by immunofluorescence. Therefore, this present result could be an additional supporting document in that, during potassium depletion, Na,K-ATPase protein still retains at the basolateral site rather than moving to the apical membrane. Indeed, the enhancement of Na,K-ATPase expression would promote K secretion through apical K channel. However, the recent study by Li et al. (2004) have demonstrated that the apical K channel is suppressed by LK intake. Therefore, K secretion would occur preferentially through basalateral site. Consequently, these secreted K ions would serve as basolateral K^+ recycling for Na,K-ATPase (Buffin-Meyer et al., 1998).

Studies of renal Na,K-ATPase during potassium depletion have demonstrated some conflicting results. Many experiments are in agreement with a number of reports showing enhanced Na,K-ATPase activity or ouabain binding produced by low ambient K in several cell lines in culture (Bowen and McDonough, 1982; Flier and Usher, 1984; Pollack et al., 1981; Pressley et al., 1986; Wardan et al., 1984) or by in vivo potassium depletion in kidney homogenates (Cronin et al., 1982; Linas et al., 1979).

By contrast, in one study of isolated rat nephron segments, a low-K diet fed to rats for 8 weeks produced a decline in Na,K-ATPase activity in cortical collecting tubules, but no change in any other segments (Garg et al., 1982). More paradoxical findings of this enzyme have been noted in microtubule segments during hypokalemia. After 3 weeks on the K-free diet, Na,K-ATPase activity was increased in CCT and MCT, but not in PCT as compared to control (Hayashi and Katz, 1987). However, some studies showed the opposite results of this enzyme activity. For instance, Na,K-ATPase activity was reduced in CCT but was enhanced in MCT during 1 to 5 weeks of low potassium diet (Imbert-Teboul et al., 1987; Eiam-Ong et al., 1993; McDonough et al.,1994; Buffin-Meyer et al., 1998; Wall et al., 1999). Meanwhile seven days of LK diet increased this enzyme activity in both PCT and isolated basolateral membrane vesicle but the protein abundance in the membrane was still remained (Eiam-Ong and Sabatini, 1999). Moreover, the recent study has demonstrated that potassium depletion for seven days increases Na,K-ATPase activity in both CCT and MCT in the younger rats, but only in MCT in the non-obese aged rats (Eiam-Ong et al., 2002).

These relevant effects of potassium depletion on Na,K-ATPase activity may be due to the duration period of potassium deficientcy and the degree of hypokalemia as well as the circulating level of aldosterone. It is

well published that aldosterone stimulates Na,K-ATPase activity and expression (Verrey et al., 1996). Therefore, hypokalemia could also reduce aldosterone secretion and consequently cause hypoaldosterone status resulting in suppression of Na,K-ATPase (Eiam-Ong et al., 1993).

Besides increased the enzyme activity, low ambient potassium could alter Na,K-ATPase subunit expression. In kidney cell lines, low potassium increased protein abundance of both α_1 and β_1 subunits (Lescale-Matys et al., 1990; Tang and McDonough, 1992; Zhou et al., 2003). Furthermore, after 16 hr-exposure of LLC-PK1/C14 cells in low potassium media, the degradation rate of $\alpha 1$ subunit was decreased as compared to control (Lescale-Matys et al., 1990). Moreover, the $\alpha 1$-and $\beta 1$-subunit mRNA abundance also were elevated by low potassium (Bowen and McDonough, 1987; Tang and McDonough, 1992). In vivo experiments, hypokalemia caused by low potassium intake for 14 days increased $\alpha 1$ and $\beta 1$ isofroms in MCT of rat both protein abundance (McDonough et al., 1994), and mRNA expression (Buffin-Meyer et al., 1998). Nevertheless, how low potassium stimulates gene expression remains to be revealed. However, one recent study has demonstrated that low potassium could induce Na,K-ATPase expression via increased reactive oxygen species (ROS) activity (Zhou et al., 2003). Since this effect of low potassium was abrogated by antioxidants, catalase and *N*-acetylcysteine (NAC) (Zhou et al., 2003). It has been shown that low potassium induced biosynthesis of the Na,K-ATPase is dependent on the intracellular iron activity that is important to generation of ROS and is inhibited by catalase (Yin et al., 2003). ROS regulate activities of several transcriptional factors, most notably NF-κB and AP-1 through enhancing c-*fos* and c-*jun* mRNA levels (Colleart et al., 1995; Webster et al., 1994). In this regard, Cayanis et al. (1992) have

demonstrated that low extracellular potassium concentration elevated c-*fos* and c-*jun* RNA abundances. The molecular effects of ten-day low potassium, in the present study, on Na,K-ATPase expression during transcription or translation remain to be investigated.

In addition, it has been shown that low potassium could alter Na,K-ATPase expression. In liver cell line experiments, low potassium medium increased Na,K-ATPase activity (Pressley et al., 1986) as well as the mRNA levels of α and β isoforms (Pressley et al., 1988). The exposure of cardiac myocytes to low potassium also increased in the abundance of α_1 and β_1 mRNA by regulatory DNA sequences in close proximity to the site of transcription initiation (Qin et al., 1994). Furthermore, low potassium also enhanced binding of transcriptional factors, sp1 and sp3, to gene promoter mediating β gene transcription in neonatal rat cardiac myocytes (Zhuang et al., 2000). By contrast, in skeletal muscle, hypokalemia induced by dietary potassium restriction for two weeks caused decreases in $\alpha2$ protein and mRNA abundance (Azuma et al., 1991). However, a prolonged hypokalemia, for three weeks, increased in the amount of the $\alpha2$ transcripts despite a decrease in its protein level (Hsu and Guidotti, 1991). Moreover, low potassium intake, for 10 days, diminished Na,K-ATPase α and β subunit protein levels with isoform and muscle type specificity (Thomson and McDonough, 1996). Of interest, the more recent study in plant subjected to potassium starvation has demonstrated that the transcript levels for the phytohormone jusmonic acid biosynthetic enzymes lipoxygenase, allene oxide synthase, and allene oxide cyclase were strongly increased (Armengaud et al., 2004).

Regarding to H,K-ATPase, in cortex, the present study shows that LK diet for 10 days increased protein expression of both $\alpha1$- and $\alpha2$-

subunit at luminal membrane of collecting tubule. In medulla, LK slightly enhanced $\alpha1$-isoform protein expression, whereas no expression of $\alpha2$ was noted. These present results are likely different from western blot analysis. Codina et al. (1998) revealed that 14 days of LK diet enhances abundance of $HK\alpha2$ protein in membranes prepared from renal medulla, but there were no changes of $HK\alpha1$ protein in either cortex or medulla. However, Kraut et al. (1997) demonstrated that the abundance of both $HK\alpha1$ and $HK\alpha2$ proteins are stimulated by potassium depletion for 14 days. The greater extent of $HK\alpha2$ expression was noted than that of $HK\alpha1$. Of interest, from northern analysis, the results showed in the same consensus that 14 days of LK diet increases mRNA level of both $HK\alpha1$ and $HK\alpha2$ (DuBose et al., 1995; Ahn et al., 1996a ; Ahn et al., 1996b; Nakamura et al., 1998; Zies et al., 2002). The renal response to potassium restriction of these two genes are likely site specific expression. Ahn et al. (1996a; 1996b) have indicated that, during chronic hypokalemia (14 days), the $HK\alpha1$-subunit gene is enhanced in renal cortex, whereas the $HK\alpha2$ isoform gene is upregulated in medullaly region. By contrast, Sangan et al. (1997) observed a decrease in the amount of $HK\alpha2$ mRNA after potassium depletion for 3 weeks. However, DuBose et al. (1995) found no change of $HK\alpha1$ mRNA expression from whole kidney.

For H,K-ATPase activity, normally, it occurs at a low level in the kidney. The alteration of this enzyme is precisely regulated by circulating potassium concentration. Hypokalemia, caused from potassium depletion for 3 days to 5 weeks, has demonstrated to increase H,K-ATPase activity in both CCT and MCT (Doucet and Marsy, 1987; Cheval et al., 1991; Eiam-Ong et al., 2002).

The undetectable expression of medullary HKα2 protein, in the present study, may imply that this protein likely expresses in this region at a very low level and ten days of LK may be not a longer hypokalemic status enough to enhance the expression. Although the antibody to HKα2 protein used in the present study could detect the expression in the cortex, or by western blot analysis (Codina et al., 1998; Kraut et al., 1997), this antibody may be not sensitive enough to detect this minute protein in the medulla. However; additional studies related to this protein level, mRNA abundance, and transcriptional factors, during ten days of LK, remain to be further investigated.

A unique finding of the present study is that, for the first time, LK could not restore the suppressive effect of V on renal Na,K-ATPase protein expression when both LK and V were administered simultaneously (Table 4). The more interesting result in the present study is that LK still plays an faithful stimulus on H,K-ATPase protein expression in the presence of V or not. The discrepancy of LK on the two ATPases remains to be encoded. Factors involving in transcription or translation during LK+V treatment may be hypothetized as mechanisms for specific and selective mode of regulation. Further investigations would account for unveil such diversities.

Of interest, in the present study, administration of vanadate alone had a very modest effect on metabolic parameters. Blood concentrations of potassium and chloride slightly decreased, meanwhile the renal function still maintained. However; when the V treated rats subjected to potassium depletion, the animal developed a progressive hypokalemia and hypochloremia, a severe azotemia, as well as salt wastage. Despite hypokalemia and more H,K-ATPase protein expression during potassium depletion, the V treated animals had a dramatic value of fractional excretion of potassium. Furthermore, the fractional excretion of chloride

and bicarbonate also were enhanced. These data suggest that renal tubular function had a marked disturbance during V+LK treatment. The precise mechanisms of these changes remain unknown. However, these obvious alterations may occur as consequences of vanadate cytotoxicity since the greater extent of vanadium accumulation in renal tissues was noted in V+LK treated rats than those of in V treated alone group. Further investigations are needed to clarify this regard.

In conclusion, the present data are the first evidence showing that LK could not restore the suppressive effect of V on renal Na,K-ATPase protein expression. Vanadate has no influence on renal H,K-ATPase protein expression. However, LK still plays an important stimulus to increase H,K-ATPase protein expression both $\alpha1$ and $\alpha2$ isoforms. Combination treatment of V and LK has profound alterations in metabolic parameter as well as renal function than those subjected to either V or LK alone.

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APPENDIX

APPENDIX

Buffer and Reagent for Immunohistochemistry

1. Buffer solution preparation

   0.2M Na,K-ATPase-KPB pH 7.4

$Na_2 HPO_4$	=	0.162 M	=	22.98	gm / H_2O
KH_2PO_4	=	0.038 M	=	5.16	gm / 1,000 ml

   4% Paraformaldehyde in 0.1 M Na,K-ATPase-Kpb pH 7.4: 100 ml

Paraformaldehyde	=	4	gm
Add H_2O	=	50	ml (in hood)

   Heat at 60˚C and stir

   Add 1N NaOH until clear in color

Add 0.2 M Na,K-ATPase-Kpb	=	50	ml

   PBS (Phosphate buffered saline) 30X conc.

NaCl	=	526	g
$NaH_2PO_4H_2O$	=	82.8	g
5 N NaOH	=	120	ml

   (40g NaOH/200 ml DW)

Add DW until	=	2,000	ml

   Working PBS pH 7.4

30X PBS	=	70	ml
DW	=	2,030	ml

2. 3% NHS in PBS pH 7.4

Working PBS pH 7.4	=	100	ml
NHS	=	3	ml

2. DAB Tris-HCl Buffer, pH 7.4

   Tris-HCl buffer, pH 7.4

      Stock A: 2.42 g Trizma base in 100 ml DW

      Stock B: 1.7 ml conc. HCl in 100 ml DW

   To make Tris-HCl buffer, pH 7.4

Stock A	= 2.5	ml
Stock B	= 2.07	ml
Add DW until	= 10	ml

   Stock DAB

      Dissolve DAB 50 mg/ml in DW

      Pipette 100 μl into microtube

      Store below 0ºC

3. Working DAB Tris-HCl buffer , pH 7.4 (made up fresh)

DAB	= 1	microtube
Tris-HCl buffer	= 10	ml
30% H_2O_2	= 10	μl

Table 5　The individual intensity score of renal Na,K-ATPase, H,K-ATPaseα1, and H,K-ATPaseα2 protein expression scored by three pathologists from 4 groups of rat: NSS+NK, NSS+LK, V+NK, and V+LK.

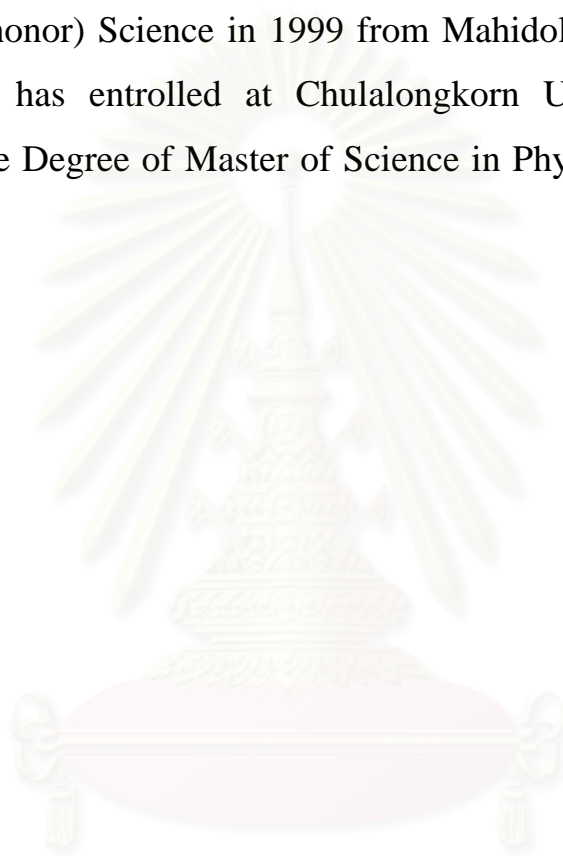| Group	#	NaK Cortex [1]	[2]	[3]	Mode	Grp	NaK Medulla [1]	[2]	[3]	Mode	Grp	α1 Cortex [1]	[2]	[3]	Mode	Grp	α1 Medulla [1]	[2]	[3]	Mode	Grp	α2 Cortex [1]	[2]	[3]	Mode	Grp	α2 Medulla [1]	[2]	[3]	Mode	Grp
NSS+NK	1	2-3	2	2-3	2-3		2-3	2	2-3	2-3		1	1	1	1		0	1	1	1		0-1	0-1	1	0-1		0	0	0	0	
	2	2	2-3	2-3	2-3	2-3	2	2-3	2-3	2-3	2-3	1	1	0	1	1	0	1	0	0	0	0	0-1	0-1	0-1	0-1	0	0	0-1	0	0
	3	3	2-3	2-3	2-3		3	2-3	2-3	2-3		1	1	1	1		0	0	0	0		0	0	1	0		0-1	0	0	0	
	4	3	2-3	3	3		3	2-3	3	3		0-1	1	1	1		0-1	0	0	0		0-1	0-1	0	0-1		0-1	0-1	1	0-1	
NSS+LK	1	3	3	3	3		3	3	3	3		1-2	2	2	2		0-1	1	1	1		2	2	2	2		1	0	0	0	
	2	3	2-3	2-3	2-3		3	2-3	2-3	2-3		2	2	1-2	2		0-1	0-1	1	0-1		2	2	1-2	2		0	0	0	0	
	3	3	3	3	3		3	3	3	3		2	2	2	2		1	1	1	1		2	2	2	2		0	0	0-1	0	
	4	3	3	3	3	3	3	3	3	3	3	1-2	2	2	2	2	0-1	1	1	1	1	2	2	2	2	2	0	0-1	0	0	0
	5	3	3	3	3		3	3	3	3		2	1-2	1-2	1-2		1	1	1	1		1-2	1-2	2	1-2		0	0	0	0	
	6	3	3	2-3	3		3	3	3	3		1-2	2	2	2		1	0-1	1	1		2	2	2	2		1	1	0-1	1	
	7	3	2-3	2-3	2-3		3	2-3	2-3	2-3		2	2	1-2	2		0-1	0-1	1	0-1		1-2	1	1	1		1	0-1	0-1	0-1	
	8	3	3	3	3		3	3	3	3		1-2	1-2	2	1-2		1	1	1	1		2	2	1-2	2		0-1	0	0	0	
V+NK	1	2	2	2	2		2	2	2	2		0-1	1	1	1		0-1	0-1	0	0-1		0-1		0-1	0-1		0	0-1	0	0	
	2	2	2	1-2	2		2	2	2	2		1	1	1	1		0	0	0	0		0-1	0-1	0-1	0-1		0-1	0	0	0	
	3	2	2	2	2		2	2	1-2	2		0	0	0-1	0		0	0-1	0-1	0-1		1	1	0-1	1		0-1	0-1	0-1	0-1	
	4	1-2	1-2	2	2	2	1-2	1-2	2	1-2	2	1	1	1	1	1	0	1	0	0	0	1	0-1	1	1	0-1	0-1	0	0	0	0
	5	2	2	2	2		2	2	2	2		1	1	1	1		0	0	0	0		0	0-1	0-1	0-1		0-1	0-1	0	0-1	
	6	1	1-2	1-2	1-2		1	1-2	1	1		0-1	0-1	1	0-1		0-1	0	0-1	0-1		1	0-1	1	1		0-1	0	0	0	
	7	2	1-2	1-2	1-2		2	1-2	2	2		0	1	1	1		0	1	0	0		0-1	0-1	0	0-1		0	0	0	0	
	8	2	2	2	2		2	2	2	2		1	0	1	1		0-1	0-1	1	0-1		0	0-1	0-1	0-1		0-1	0-1	0	0-1	
V+LK	1	1	2	2	2		1-2	2	2	2		2	2	2	2			0	0-1	0		0-1	2	2	2		0	0	0	0	
	2	2	2	2	2		2	2	2	2		1-2	2	1-2	1-2		0-1	1	1	1		2	2	2	2		0	0-1	0	0	
	3	2	2	2	2		2	2	2	2		2	2	2	2		1	1	0	1		1	1	2	1		0-1	0	0	0	
	4	2	1-2	1-2	1-2	2	2	1-2	2	2	2	2	2	2	2	2	0-1	0	0	0	1	2	1	2	2	2	0	0-1	0	0	0
	5	2	2	2	2		2	2	2	2		1-2	2	2	2		1	1	1	1		2	2	2	2		0-1	0	0-1	0-1	
	6	1	1	1-2	1		1	1-2	1-2	1-2		1-2	1-2	1	1-2		0-1	1	0-1	0-1		1-2	2	2	2		0-1	0	0	0	
	7	2	2	2	2		2	2	2	2		1	1	1	1		1	1	1	1		1-2	2	2	2		0	0	0	0	
	8	1-2	1	1-2	1-2		1	1	1-2	1		1	1-2	1	1		1	1	11	1		2	1-2	1-2	1-2		0-1	0-1	0	0-1	

Describing Data (The mode)

The mode is the value that occurs most frequently. Even nonnumerical data, for example, the immunohistochemistry staining scores or pathological scores, these scores can have a mode. There, considering the full set of 8 samples, in the present study, immunohistochemistry staining scores in each animal from each group were shown in Table 5.

BIOGRAPHY

Miss. Ratanaporn Jerawatana was born on September 17, 1977 in Saraburi province, Thailand. She received the Bachelor degree of Nursing (second class honor) Science in 1999 from Mahidol University, Bangkok, Thailand. She has entrolled at Chulalongkorn University in graduate program for the Degree of Master of Science in Physiology and graduated in 2004.