CHAPTER V

THE EFFECT OF INTRAVENOUS INFUSION OF STEVIOSIDE ON THE URINARY SODIUM EXCRETION

Introduction

Little is known about the effect of SVS on the physiological function particulary in relation to renal function, although SVS and aqueous extracts of Stevia has been shown to induce pronounced decreases in blood pressure and heart rate (Boech and Humboldt, 1981). The effect of intravenous infusion of SVS on the renal function especially natriuresis, kaliuresis, diuresis and renal vasodilation without changes of glomerular filtration rate (GFR) has been found in chapter IV. It has been suggested that its action is similar to several vasodilators such as prostaglandin (Baer, 1988). The real site and mechanisms of action of SVS in hypotension and natriuresis have not yet been elucidated. The present experiment was therefore undertaken to examine the possible mechanism of natriuretic effect of SVS infusion. Since proximal renal tubule is known to be the vulnerable site for several toxic agents, the natriuresis induced by SVS infusion may be its inhibitory action on proximal tubular Na^+ reabsorption. To clarify this possibility, lithium clearance (C_{Li}) method which has been used for evaluation of Na^+ and H_2O delivery from proximal tubule (Thomsen,1990), was performed in the present study. Moreover, enzyme activity of Na^+,K^+ ATPase, the key enzyme for renal tubular reabsorption of sodium and

potassium ; renal mitochondrial activity which is the source of energy supplying normal function of Na^+, K^+ ATPase; have been also performed during SVS administration.

Materials and Methods

Animal preparation

Male Wistar rats weighing 250-300 g were used. The animal was anesthetized by intraperitoneal injection of either sodium pentobarbiturate (45 mg/kg.BW) or inactin (100 mg/kg.BW) according to the experimental design. A rectal probe was inserted in order to continuously maintain body temperature 37.5°C. After tracheostomy, both femoral veins and arteries were canulated to infuse solution and collected arterial blood respectively. Both right and left ureters were canulated to collect urine sample. The experiments were performed as described in experimental procedures.

Experimental procedures

Experiments were divided into 3 trials.

Trial I Determination for the site of SVS's natriuretic action

The reabsorption of sodium (Na^+) and water (H_2O) along the proximal tubule but not to any measurable degree in more distal nephron segments has been determined by using lithium clearance (C_{Li}) technique. Many lines of evidences supporting this hypothesis have been reviewed by Thomsen (1990).

At the beginning of the experiment, inulin solution (1 g /100 ml normal saline) was infused intravenously at the rate of 10 ml/hr/kg.BW. and continued throughout the experiment. After 40 min, LiCl solution (0.015 M) was infused at the rate of 10 ml/hr/kgBW throughout the experiment after given of a bolus dose of 0.20 ml of 0.07 M LiCl solution. By this manoeuver, plasma Li concentration could be maintained at the level of 0.2-0.4 mEq/L. After LiCl administration for 10 min, the experiment was started. The experiment was divided into five 30-min periods; control period, the first and the second of SVS infusion periods, and the first and the second of recovery periods. Urine collection from both ureteres was carried out during every 30-min period to obtain a measure of urine flow rate (V). Arterial blood sample (0.5 ml) was collected at the mid point of each urine collection. After the control period, 0.15 g of SVS in 1 ml of normal saline (NSS) was injected intravenously in a dose of 200 mg/kg.BW and followed by continuous infusion at the rate of 200 mg/kg.BW/hr. for 1 hour. Glomerular filttration rate (GFR) was determined by inulin clearance (C_{In}). Urine and plasma sodium and lithium concentrations were measured by Flame Photometer to calculate the following equations.

Reabsorption of water in proximal tubule = $C_{In} - C_{Li}$

Reabsorption of sodium in proximal tubule = $(C_{In} - C_{Li}) P_{Na}$

Reabsorption of water at distal nephron = $C_{Li} - V$

Reabsorption of sodium at distal nephron = $(C_{Li} - C_{Na}) P_{Na}$

Delivery of sodium out of proximal tubule = $C_{Li} = C_{Na\ prox}$

The fractional excretion of sodium from proximal tubule = $C_{Li}/C_{In} = FE_{Li} = FE_{Na\ prox}$

Urinary excretion of sodium expressed as fraction of amount delivered from proximal tubule = C_{Na}/C_{Li}

Urinary excretion of water expressed as fraction of amount delivered from proximal tubule = V/C_{Li}

Trial II Determination of renal Na^+, K^+ ATPase activity

In this trial, animals were divided into two groups, the control group and a group of SVS infusion. After animal preparation, SVS solution (0.15 g/ml) was injected in a dose of 200 mg/kg.BW via the femoral vein and followed with a continuous infusion at the rate of 200 mg/kg.BW/hr. In the control group, NSS was infused with the rate of 1%kg.BW instead of SVS. After 30 min of either SVS or 0.9% NSS administration, both kidneys were quickly removed and washed using ice-cold medium as suggested in chapter III. The kidneys were decapsulated and the renal cortex from each half part of both kidneys was separated and the remaining half was pooled to determine both total renal and cortical Na^+, K^+ ATPase activity as described in chapter III. Enzyme activity was expressed as μmole of Pi released/mg protein/hour.

Trial III Determination of renal mitochondrial activity.

In this trial, two groups of experiments were carried out as the control group and experimental group of SVS infusion. All animals in this experiment were anesthetized by intraperitoneal injection of inactin (100 mg/kg.BW) by the reason described in chapter III. Animal preparation in this trial was the same as used in the

experiment to determine Na^+, K^+ ATPase activity. In the experimental animal, 200 mg/kg.BW of SVS solution (0.15 g/ml) was intravenously infused as a priming dose and then followed by continuous infusion at the rate of 200 mg/kg.BW/hr for 30 min. This time interval was chosen because of the highest response on renal function. In the control group,NSS was infused at the rate of 10 ml/hr/kg.BW. After 30 min of infusion, renal mitochondrial preparation was accomplished using method described in chapter III.

After the preparation, mitochondria was pored into the incubation medium. The substrate was added and state 4 of respiration was measured. 600 nM of ADP in the presence of glutamate (5 mM) plus malate (5 mM) or 300 nM in the case of succinate was added to initiate state 3 respiration. The ratio of Vo_2 in state 3 to state 4, termed respiratory control ratio (RCR) was determined as well as ADP:O or P/O ratio. Renal mitochondrial ATP synthetase activity was also evaluated.

Statistics

The statistical analysis was performed using both paired and unpaired t-test. the significant level was determined at $P<0.05$. All values are expressed as mean ± SEM. Linear correlation was also performed.

Results

Effect of SVS on lithium clearance and renal tubular sodium reabsorption

Data in table 5.1 demonstrates that urine flow rate (V) significantly increased during SVS infusion (53.6 % in the first period and 31.2 % in the second period) and

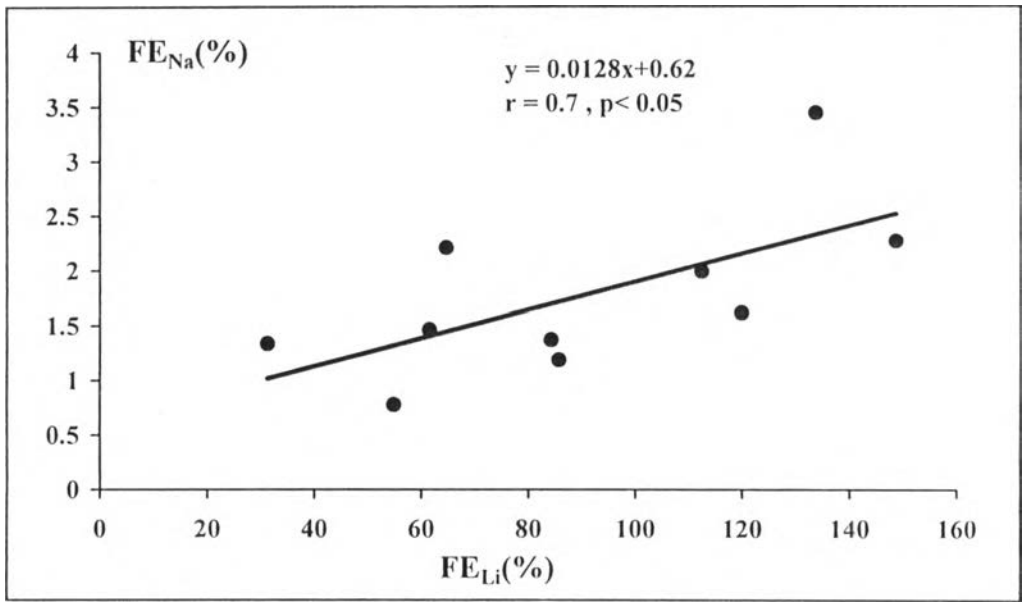
Table 5.1 The effects of SVS infusion on lithium clearance as a marker of proximal tubular Na^+ reabsorption.

Parameters \ Period	control	SVS infusion period		recovery period	
	30 min	60 min	90 min	120 min	150 min
V (μL/min)	21.5±3.3	33.0±3.5***	28.2±2.8**	27.3±2	25.4±2.9
GFR (ml/min/kg.BW.)	6.1±0.5	5.9±0.5	5.2±0.4	4.7±0.4*	4.8±0.3*
$C_{Li} = C_{Naprox}$ (μL/min/kg.BW.)	2449±177	5298±836**	2623±346	2171±306	2291±337
$V/C_{Li} = V/V_{prox}$ (%)	3.3±0.5	2.6±0.3	4.5±0.7	5.3±0.6*	4.6±0.5
$FE_{Naprox} = FE_{Li}$ (%)	42.3±4.0	89.7±12.0**	52.6±7.8	45.8±5.1	47.3±5.2
C_{Na} (μL/min/kg.BW.)	55.7±11.9	104.4±15.4***	86.4±13.3***	80.5±8.1**	76.4±12.4*
FE_{Na} (%)	0.9±0.2	1.8±0.2***	1.6±0.2***	1.7±0.1**	1.6±0.2**
C_{Na}/C_{Li} (%)	2.2±0.4	2.7±0.3	3.7±0.6*	4.2±0.6**	3.7±0.4*
prox H_2O reabsorption (μL/min/kg.BW.)	3597±407	605±691**	2564±455	2507±251*	2464±244*
prox Na^+ reabsorption (μEq/min/kg.BW.)	510.5±57.3	81.97±97.6**	359.7±63.2	356.9±37.3*	345.6±31.5*
Distal H_2Oreabsorption (μL/min/kg.BW.)	2367.8±169.7	5174.2±822.9**	2518.0±340.0	2070.1±299.1	2197.5±327.6
Distal Na^+reabsorption(μEq/min/kg.BW.)	339.6±22.1	743.8±121.2**	363.3±51.7	299.5±45.2	315.1±48.4

Values represent mean ± S.E.M.(n = 10)

Abbreviations : V,urine flow rate;C_{Li}, clearance lithium ; FE_{Li}, fractional excretion of lithium ; C_{Na} ,clearance of sodium ; FE_{Na}, fractional excretion of sodium ;C_{Na}/C_{Li} ,V/C_{Li} urinary excretion of sodium and water expressed as fractions of amount delivered from proximal tubule; FE_{Naprox} fractional excretion of sodium from proximal tubule. Significant differences comparing to control period using paired t-test are indicated by *P<0.05, **P<0.01,***P<0.001.

A



B

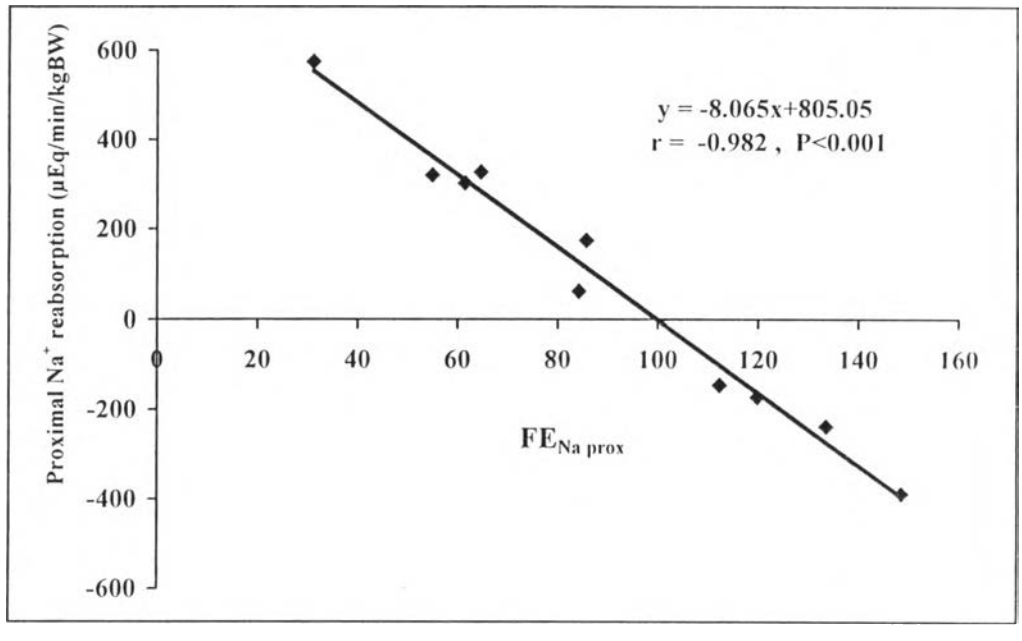


Figure 5.1 The relationship between FE_{Na} versus FE_{Li} (A), and proximal Na^+ reabsorption versus FE_{Naprox} (B) . Data are derived from the results during the first period of SVS (200 mg/kg.BW/hr) infusion.

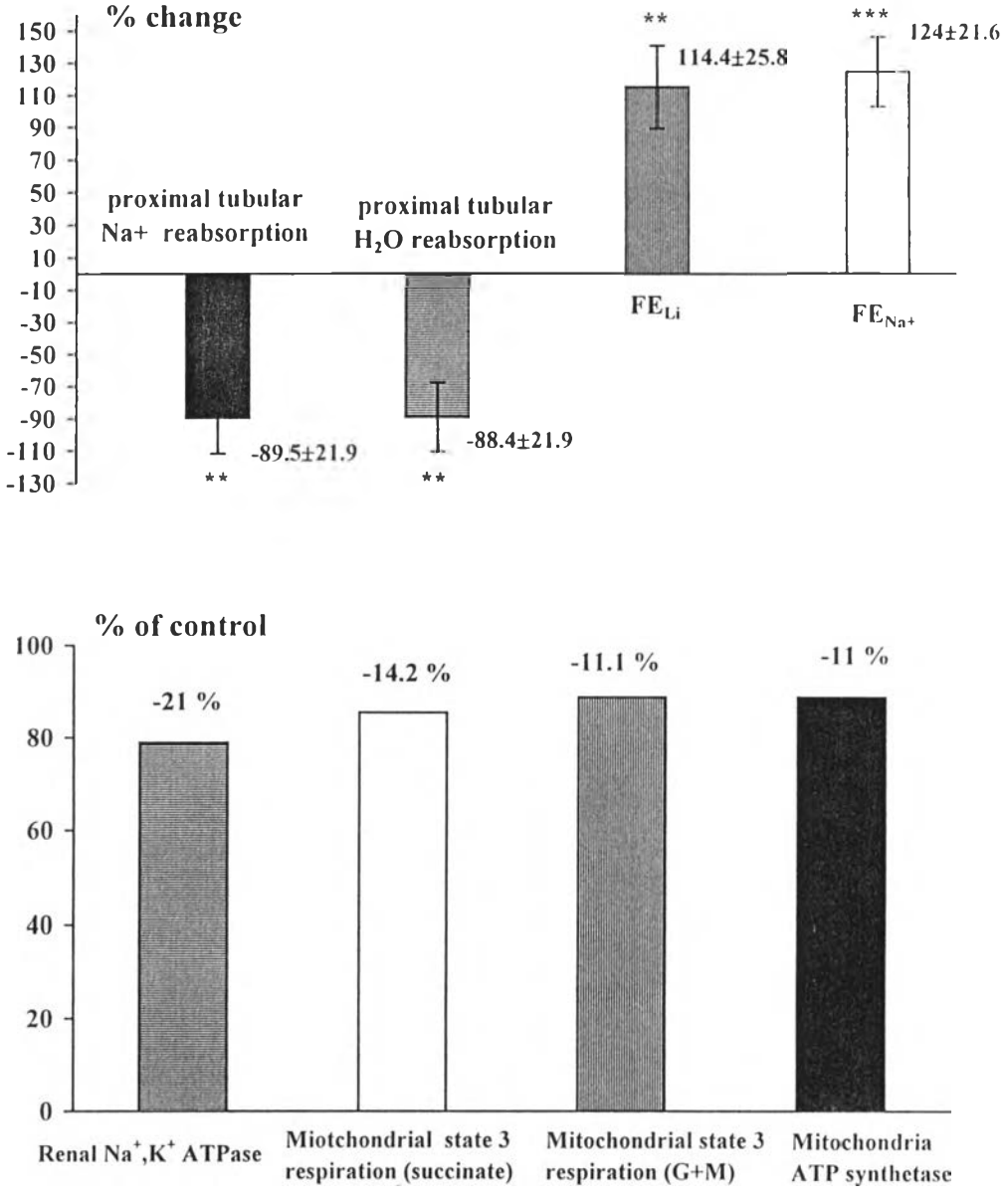


Figure 5.2 The alteration of proximal tubular reabsorption of Na^+ and H_2O, FE_{Li}, FE_{Na}, (upper panel), renal Na^+,K^+ ATPase and renal mitochondrial activity using succinate and glutamate plus malate (G+M) as substrate in response to SVS infusion.

then returned to normal level after the end of SVS administration. GFR was not affected during infusion of SVS but it significantly reduced in the recovery period. Plasma lithium concentration was average 0.2-0.4 mEq/L throughout experiment. Lithium clearance (C_{Li}) or C_{Naprox} representing delivery of sodium out of proximal tubule significantly increased from 2449±177 to 5298±836 μL/min/kg.BW ($P<0.01$) in the first period of SVS infusion. During the second period of SVS infusion, C_{Li} was still high but not significantly different from the control period. No changes of C_{Li} were apparent in recovery periods. Similarly, FE_{Li} or $FE_{Na\ prox}$, the representation of the fractional excretion of sodium from proximal tubule, markedly increased from 42.3±4.0 to 89.7±12 % ($P<0.01$) during SVS administration. Slight elevation but not significant difference from the control of FE_{Li} was apparent during first period of SVS infusion and recovery periods. FE_{Na} significantly increased throughout experimental periods. Proximal tubular water and sodium reabsorptions significantly reduced ($P<0.01$) as a consequence of first period of SVS infusion. Figure 5.1 A shows the relationship between FE_{Na} and FE_{Li} during the first period of SVS infusion. There was a close relationship between both parameters ($\gamma=0.7$, $P<0.05$). There was a highly negative correlation between proximal tubular Na reabsorption and $FE_{Na\ prox}$ ($\gamma=0.98$, $P<0.001$).(figure 5.2 A)

The reduction of proximal tubular water and sodium reabsorption was apparent by approximately 40% ($P<0.05$) during recovery period. At distal nephron, water reabsorption was significantly increased from 2368±170 to 5174±823 μL/min/kg.BW ($P<0.01$) and sodium reabsorption significantly increased from 340± 22 to 744±121 μEq/min/kg.BW($P<0.01$) during first period of SVS infusion. No

significant alterations took place in both sodium and water reabsorption at distal nephron beyond this period. The urinary excretion of sodium and water as the fractions of amount delivered from proximal tubule estimating by C_{Na}/C_{Li} and V/C_{Li} (V/V_{prox}) respectively, were unaltered in the first 30 min of SVS infusion but it markedly increased in the latter period.

Na^+, K^+ ATPase activity in response to SVS infusion.

The effect of SVS infusion on Na^+, K^+ ATPase activity in either whole kidney or renal cortex is shown in table 5.2 . There were no significant changes of total ATPase activity in either whole kidney or renal cortex between the control group and SVS infusion group. Similar results were apparent for Mg^{2+} ATPase activity. However, Na^+, K^+ ATPase activity was significantly declined from 26±1.8 to 20.5 ± 1.4 μmole Pi/mg protein/hr ($P<0.05$) in the whole kidney and 33.3±2.8 to 26.2±1.3 μmole Pi/mg protein/hr ($P<0.05$) in the renal cortex after SVS infusion.

Effects of SVS on renal mitochondrial function

The effect of SVS on the renal mitochondrial activity is shown in table 5.3. Both site I (using glutamate+malate as substrate) and site II (using succinate as substrate) of mitochondrial activity were determined. With glutamate+malate as substrates, control mitochondria had substrate-supported oxygen consumption (Vo_2) of 13.6±0.4 ng atom O_2/min/mg protein which was not significantly different from that of SVS infusion group (12.8±0.9). During state 3 respiration, ADP addition, Vo_2 was stimulated to 97.1± 4.3 in control group and 86.3±5.9 ng atom O_2/min/mg protein

in SVS infusion group. Mitochondrial activity in response to SVS administration had a low RCR from 7.2±0.2 to 6.9±0.2 but not statistical difference. SVS infusion caused a significant decreased in total Vo_2 after ADP application ($P<0.05$) and then resulting a significant elevation of the P/O ratio from 3.5±0.2 to 4.4±0.3 ($P<0.01$).

With succinate as a substrate, the same result was obtained. SVS infusion had no influence on state 4 respiration whereas significant decreased of state 3 respiration from 165.7± 9.9 to 142.1±7.1 ng atom O_2/min/mg protein ($P<0.05$) was obtained. SVS depressed total Vo_2 by 22.8% ($P<0.01$) which then resulted the significant increase of P/O ratio from 2.3±0.1 to 2.8±0.1 ($P<0.01$). Renal mitochondrial ATP synthetase activity was decreased from 2.8±0.1 to 2.5±0.1 μmole Pi/mg protein/hr ($P<0.05$) after SVS infusion in comparing with the control animal.

The reduction of state 3 respiration was only 14.2% with succinate and 11.1% with glutamate + malate as substrate (figure5.2). Moreover, mitochondrial ATP synthetase activity was slightly decreased by approximately 11%. However, the depression of proximal Na^+ and H_2O reabsorption was markedly high (88%) (figure 5.2).

Discussion

To evaluate the effect of SVS infusion on proximal renal tubular function, C_{Li} method was performed. This method has been developed and used by several investigators. The reliability of C_{Li} method was discussed by Thomsen (1990) and

Table 5.2. Effect of SVS infusion on renal Na^+, K^+ ATPase activity.

Group / Parameters	control (N = 9)	SVS infusion (N = 9)
renal cortex		
Total ATPase (μmol Pi /mg prot /hr)	53.9±2.9	48.1±3.7
Mg^{2+}ATPase (μmol Pi /mg prot /hr)	20.7±1.7	21.6±3.5
Na^+,K^+ATPase(μmol Pi /mg prot /hr)	33.3±2.8	26.2±1.3*
whole kidney		
Total ATPase (μmol Pi /mg prot /hr)	48.6±1.0	46.6±2.4
Mg^{2+}ATPase (μmol Pi /mg prot /hr)	22.6±1.2	26.1±2.3
Na^+,K^+ATPase (μmol Pi /mg prot /hr)	26.0±1.8	20.5±1.4*

Values represent the mean±S.E.M. Enzyme activity was expressed as μmole Pi released per mg protein per hour (μmol Pi /mg prot /hr). The significant differences between control and SVS infusion group are determined using unpaired t-test : *P< 0.05.

Table 5.3 Changes of renal mitochondrial activity comparing between control and SVS infusion group.

Parameters \ Group	control (N = 9)	SVS infusion (N = 9)
succinate as substrate		
St 4 (ngatom O_2 /min/mg prot)	37.8±2.0	35.7±1.6
St 3 (ngatom O_2 /min/mg prot)	165.7±9.9	142.1±7.1*
total O_2 consume (ngatom O_2/mg prot)	106.9±7.8	82.5±6.1*
RCR	4.4±0.1	4.0±0.1**
P/O	2.3±0.1	2.8±0.1**
glutamate+malate as substrate		
St 4 (ngatom O_2 /min/mg prot)	13.6±0.4	12.8±0.9
St 3 (ngatom O_2 /min/mg prot)	97.1±4.3	86.3±5.9
total O_2 consume (ngatom O_2/mg prot)	142.1±11.6	108.0±9.0*
RCR	7.2±0.2	6.9±0.2
P/O	3.5±0.2	4.4±0.3**
ATP synthetase activity(μmol Pi/mg prot/hr)	2.8±0.1	2.5±0.1*

Values represente mean±S.E.M. Abbreviations : St 3, St 4 oxygen uptake in state 3 and 4 of mitochondrial respiration ; RCR, respiratory control ratio ; P/O, ratio of ADP added to total O_2 uptake during state 3 of mitochondrial respiration ; ATP synthetase activity, mitochondrial ATPase synthatase activity. Significant differences between the control group and SVS infusion group are determined using unpaired t-test were determined by *$P<0.05$, **$P<0.01$.

Leyssac (1990). By comparing C_{Li} method to the method using either micropuncture or transit time-occlusion time, there was a close relationship between proximal tubular lithium reabsorption and proximal tubular reabsorption of Na^+ and H_2O (Thomsen, 1990). In these experimental results, the elevation of urine flow and urinary electrolyte excretion in response to SVS administration has been noted. Significant increases in C_{Na} and FE_{Na} were associated with the elevation of C_{Li} and FE_{Li} during the first period of SVS infusion. These results indicate that SVS could affect proximal tubular reabsorption of Na^+. The fractional urinary excretion of Na^+ and H_2O in relation to amount delivered from proximal tubule as expressed by C_{Na}/C_{Li} and V/C_{Li} remained unaltered, suggesting that the elevation of urinary Na^+ and H_2O excretion could be the predominant consequence effect of SVS infusion. The increase of filtered load would not be the case since GFR and plasma Na^+ concentration were unchanged. The enhancement of reabsorption of Na^+ and H_2O in distal nephron (table 5.1) may be the compensatory response to the proximal tubular Na^+ loss. The mechanism may be that hypotensive effect of SVS may activate sympathetic activity which then results the stimulation of renin-angiotensin-aldosterone system to increase Na^+ reabsorption. However, the facilitation of distal tubular reabsorption of Na^+ induced by aldosterone is unlikely because it requries time, at least 90-180 min (Reif et al, 1986). SVS infusion has been found to induce hypotension (Melis,1992a). Therefore, Arginine vasopressin liberation in response to hypotension (McNeill, 1983), and thereby enhance Na^+ and H_2O reabsorption at the thick ascending limb of loop of Henle and collecting tubule would be possible (Koeppen and Stanton,1992).

During the second period of SVS infusion, the same pattern response was remained taken place but to a lesser extent. This may be because some portion of infused SVS remained in the body (Ishü and Bracht,1995). It was interesting that C_{Na} and FE_{Na} in the recovery period were still increased even less than that of SVS infusion period. However, GFR was significantly declined while C_{Li} and FE_{Li} returned back to base-lined level, thereby caused the significant rise of C_{Na}/C_{Li}. The proximal tubular reabsorption of Na^+ and H_2O was improved but slightly less than that of control level. The tubular reabsorption at distal nephron was not changed. Therefore, the remaining elevation of Na^+ and water excretion during recovery period should be the result from the diminution of proximal tubular reabsorption. It is possible that infused SVS might not be totally excreted, therefore some portion of SVS could act on tubular cell (Ishü and Bracht,1995).

It is widely known that movement of $Na^+$and K^+ across tubular cell membrane is mainly dependent on the normal function of enzyme Na^+, K^+ ATPase linning at the basolateral membrane of renal tubular cells. In rat, an abundant of Na^+, K^+ ATPase has been shown to present at the proximal tubule (Doucet, 1992). In the present study, SVS infusion caused natriuresis and diuresis by inhibition of reabsorptive function on proximal tubule. However, no evidence supports the mechanisms of its inhibitory action. SVS infusion decreased enzyme Na^+, K^+ ATPase activity in both renal cortex and the whole kidney (table 5.2). The same proportion in the reduction of enzyme activity in both renal cortex and the whole kidney was apparent. These findings indicate that natriuretic effect of SVS infusion

was the result from the impairment of proximal tubular Na^+, K^+ ATPase activity. SVS administration may directly disturb enzyme activity and/or energy supply. The present experiment for mitochondrial activity demonstrated that state 4 respiration was unaltered reflecting undisturbance of SVS on inner mitochondrial membrane permeability. The reduction of oxgen consumption (Vo_2) during state 3 respiration (ADP-stimulated respiration) was obtained with glutamate+malate and succinate as substrate. Likewise, total Vo_2 following ADP addition was significantly depressed even either succinate or glutamate+malate used for substrates, resulting the significantly augmented P/O ratio. The reduction in ADP-stimulated respiration may reflect the defects in either the electron transport chain, ADP-ATP translocator, ATP synthetase activity or some combinations of the above (Malis and Bonventre, 1986). The lowered mitochondrial ATP synthetase activity after SVS infusion was noted. It indicates that at least the impairment of mitochondrial ATP synthetase activity was one of the possible factors to cause the reduction of state 3 respiration. In addition, site II (succinate as substrate) is more susceptible site for SVS interfering action on mitochondrial respiratory activity because of the lower value of state 3 respiration when comparing to that of site I. The decrease in Vo_2 in the presence of ADP and the interuption of ATP synthetase activity occured in response to SVS infusion. This may be explained at least the defect of oxidative phosphorylation to generate ATP during SVS administration. The disturbance of renal mitochondrial function in response to SVS infusion was similar to the result studying in isolated rat liver mitochondria (Bracht et al., 1985a). In the present study, general bleeding was observed in renal tissue following 30-min period of SVS infusion. Thus, the lower mitochondrial

activity and Na^+, K^+ ATPase activity may be the result from direct influence of SVS and/or renal ischemia. The reduction of state 3 respiration and RCR value with either succinate or glutamate/malate as substrate in response to renal ischemia has been reported (Jung and Pergande,1988). Furthermore, renal cortical tissue and site II substrate utilization were more vulnerable to ischemia. This indicates that the degradation of mitochondrial respiratory function during SVS infusion may be ,in part, affected by renal ischemia.

Several lines of evidences have indicated that the inhibition of Na^+, K^+ ATPase brought about the decline of state 3 respiration, and the damage of renal mitochondria resulted the depression of this enzyme activity (Harris et al., 1981; Soltoff and Mandel, 1984). However, it can not be just from our experimental results which phenomena between the interuptive action of SVS on mitochondrial function and SVS'effect on enzyme activity would be appeared first. Interestingly, SVS infusion suppressed mitochondrial function and Na^+, K^+ ATPase activity around 14 and 21% respectively whereas inhibited proximal tubular Na^+ and H_2O reabsorption approximately 88% during the first 30 min of SVS infusion. This may indicate that there were disturbances of tubular reabsorptive cell other than the depletion of Na^+, K^+ ATPase activity and mitochondrial function during SVS infusion.

In conclusion, SVS infusion produces natriuresis and diuresis by reducing the proximal tubular reabsorption of Na^+ and H_2O. The reduction of renal mitochondrial and Na^+, K^+ ATPase activity would account for the loss of Na^+ and H_2O during SVS infusion. However, the magnitude of the reduction of both renal mitochondrial and

Na^+, K^+ ATPase activity did not totally explain the high elevation of Na^+ excretion during SVS infusion. Other factors such as its direct effect on tubular reabsorptive ability may be possible . SVS has been reported to shortening microvilli and damaged brush border of proximal tubular convoluted cell (Toskulkao et al, 1994).