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Effects of Hydrochlorothiazide on the Renal Cyclic Nucleotides Level

Seok-Yong Lee,* Taek-Lip Koh, Woo-Young Lee, Sang-Bok Lee and Kyu-Chul Cho

Department of Pharmacology, Catholic Medical College, Seoul 135, Korea

ABSTRACT

To determine the relationship between hydrochlorothiazide-induced diuretic action and cyclic nucleotides, the effects of hydrochlorothiazide (5 mg/kg, i.v.) on the renal tissue level of cyclic nucleotides and the renal adenylate cyclase and guanylate cyclase activity were investigated. Hydrochlorothiazide elecitied the maximal diuretic effect between 10 and 20 min after the injection of drug. The increased urine flow and urinary electrolytes excretion returned to the control levels 60 min after the injection of drug. 5 and 15 min after drug administration the cAMP level of renal tissue was significantly decreased, but 60 min after the cAMP level was not different from the control level. The cGMP level of renal tissue was not affected by hydrocholorothiazide. Hydrochlorothiazide (5×10^{-4} M) inhibited the renal adenylate cyclase but not affected the renal guanylate cyclase. These results suggest that cAMP may be involved in the renal action mechanism of hydrochlorothiazide and the involvement of cGMP is uncertain.

Key Words: Hydrochlorothiazide, cAMP, cGMP, adenylate cyclase, guanylate cyclase

INTRODUCTION

Hydrochlorothiazide has been used extensively as a diuretic and antihypertensive agent. The diuretic effect of the thiazides is largely attributed to inhibition of the active transport of sodium from the tubules into the blood, but the precise mechanism of their action is not yet fully understood. Since the thiazides do not affect the corticomedullary osmotic gradient, they are considered not to interere with sodium reabsorption in the ascending limb of the loop of Henle (Burg, 1981). The some members of thiazides significantly inhibited carbonic anhydrase (Beyer, 1958; Maren, 1967) and Wilson *et al.*, (1983) observed the inhibitory effect of hydrochlorothiazide on chloride, sodium and water reabsorption in the medullary collecting duct of rat. But, these effects are considered relatively unimportant in the diuretic response (Kunau *et al.*, 1975; Bowman & Rand, 1980). The thiazides have an important site of action in the distal convoluted tubule and inhibit the active transport of sodium in this portion (Fernadez & Puschett, 1973; Edwards *et al.*, 1973; Constanzo & Windhager, 1978).

Very little is known about the mechanism by which sodium transport is inhibited by thiazides. Recently, Stokes (1984) demonstrated an inhibitory effect of hydrochlorothiazide on a simple electrically neutral NaCl cotransport system in the urinary bladder of the winter flounder. This system was shown to be responsive to thiazide analogs. But, it has not been determined if a similar transport mechanism is present in the distal convoluted tubule, and the mechanism through which the thiazides interact with the NaCl transport system is unclear.

Some studies on the interrelationship between the renal electrolytes reabsorption and the cyclic nucleotides have been reported. cAMP inhibited a carbonic anhydrase (Beck *et al.*, 1975; Mckinney & Myers, 1980) and the reabsorption of electrolytes and water in the proximal tubule (Hamberger *et al.*, 1974; Weinman *et al.*, 1984), but cAMP increased the Cl^--conductance in the thick ascending limb of

* To whom reprint requests should be addressed

Henle's loop (Schlatter & Greger, 1985) and the permeability of water in the collecting duct (Grantham & Orloff, 1966; Grenier *et al.*, 1982). Osswald (1974) demonstrated that cGMP injected into the thoracic aorta of rats induced an increase of the renal electrolyte excretion. Lee and Cho (1983), and Lee *et al.*, (1985) proposed that furosemide action may be related to the increase of the renal tissue level of cGMP. However, the relationship between the renal effect of hydrochlorothiazide and the cyclic nucleotides was not reported.

In the present study, the effects of hydrochlorothiazide on the renal tissue level of cyclic nucleotides and the renal adenylate and guanylate cyclase activity were investigated.

MATERIALS AND METHODS

Chemicals

Hydrochlorothiazide was purchased from Ciba-Geigy (Switzerland), inulin from Difco Lab. (U.S.A.), PAH from Metheson Coleman & Bell Co. (U.S.A.) and ATP, GTP, creatine phosphate, creatine phosphokinase and theophylline from Sigma (U.S.A.).

Clearance experiment

Male rabbits, 2.0-2.2 kg, were used. Intraperitoneal urethan (1.2 g/kg) was used for anesthesia in nonfasted animals. Following tracheotomy, the left femoral vein was cannulated with polyethylene tube (22 G.) for the infusion (0.5 ml/kg/min) of isotonic saline containing 0.3% inulin and 0.04% p.aminohippuric acid (PAH), and the left femoral artery was cannulated with polyethylene tube (22 G.) for blood-sampling. Through a small abdominal incision, the left and right ureters were cannulated with polyethylene tube (23 G.) for urine collection. Thirty minutes after three 10-min urine collections were performed and then hydrochlorothiazide (5 mg/kg) was injected intravenously. After injection of drug six 10-min urine collections were performed. A midpoint blood of each collection time was withdrawn. Sodium and potassium concentrations in urine were measured with flame photometer (Instrumentation Lab., U.S.A.). Chloride concentration in urine was measured with chloridometer (Buchler-Coltlove Instrumentation, U.S.A.) and osmolarity with osmometer (Precision Osmometer Inc., U.S.A.). Inulin and PAH concentrations in urine and plasma were measured by methods of Schreiner (1950) and Smith *et al.*, (1945), respectively.

Animal experiment for cAMP and cGMP assay

Male Wistar rats, 180-200 g, were used. They were fasted 12 h prior to the experiment while having free access to water. The animals were anesthetized with secobarbital 30 mg/kg i.p. and given hydrochlorothiazide 5 mg/kg or isotonic saline 0.5 ml/Kg intravenously. 0, 5, 15 or 60 min after injection of the drug the kidney were quickly removed and frozen with dry ice.

Tissue preparation for cAMP and cGMP assay

The frozen kidney were homogenized in 10 ml of 0.7 N perchloric acid. The homogenate was centrifuged at 10,000g for 15 min and the supernatant was collected. The precipitate was solubilized in 1 N NaOH and assayed for protein. Protein assay was performed by the method of Lowry *et al.*, (1951). The collected supernatant was neutralized with 5 N potassium hydroxide and the precipitate removed by centrifugation. An aliquot of this supernatant was lyophilized and redissolved in 50 mM Tris/EDTA buffer (pH 7.4) for cAMP and cGMP assay. Tissue levels of cAMP or cGMP were expressed as pmoles/mg protein.

Assay of cAMP and cGMP

Assay of cAMP and cGMP was performed by radioimmunoassay with cAMP RIA kit and cGMP RIA kit (Amersham, England).

cAMP assay: The incubation mixture comprised (^{3}H)-cAMP, binding protein, sample or standard cAMP, and 50 mM Tris/EDTA buffer (pH 7.4) in a final volume of 200 μl. The tubes were kept at 4°C for 2 hr. After incubation, in order to remove the unbounded cAMP and (^{3}H)-cAMP 100 μl of the charcoal suspension was added to each tube. The tubes were then briefly agitated and immediately centrifuged at 400g for 10 min at 4°C. 200 μl of the supernatant was then taken into the scintillation vial contained 10 ml scintillant mixture for counting. The scintillant mixture was composed of 667 ml toluene, 333 ml Triton X-100, 7 g PPO and 0.3 g POPOP.

cGMP assay: The incubation mixture comprised (^{3}H)-cGMP, antiserum, sample or standard cGMP, and 50 mM Tris/EDTA buffer (pH 7.4) in a final volume of 200 μl. The tubes were kept at 4°C for 1.5 hr. After incubation, 1 ml ice-cold ammonium

sulphate solution was added to each tube. Five minutes after the tubes were centrifuged at 400g for 10 min at 4°C. After centrifugation the precipitate was resolved in 1.1 ml distilled water. 1 ml of this solution was placed in the scintillation vial with 10 ml scintillant mixture for counting.

Preparation of adenylate cyclase and guanylate cyclase

Male Wistar rats (180-200 g) were killed by decapitation. The kidneys were quickly removed and immersed in 0.25 M sucrose. The renal tissue was blotted, weighed and homogenized in 9 volumes (W/V) of ice-cold homogenizing buffer (0.25 M sucrose, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM dithiotheritol) with 10-15 strokes using glass homogenizer and motor driven Teflon pestle. The homogenate was centrifuged at 600g for 10 min. The supernatant was recovered and centrifuged at 8500g for 15 min. The resulting precipitate was washed twice with 200 mM Tris-HCl (pH 8.8), 0.25 M sucrose, 1 mM dithiotheritol and 5 mM $MgCl_2$, and then suspended in same buffer (4-6 mg/ml). This suspension was used for assay of adenylate cyclase activity. The supernatant recovered after centrifugation at 8500g was recentrifuged at 105,000g for 60 min. The supernatant was used for assay of guanylate cyclase activity. Protein was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin as standards.

Assay of adenylate cyclase

The assay medium contained 50 mM Tris-HCl (pH 7.6), 10 mM theophylline, 15 mM creatine phosphate, 20 μg creatine phosphokinase (100-150 units/mg), 5 mM $MgCl_2$, 1 mM ATP and enzyme preparation (40-60 μg protein) in a final volume of 100 μl. After 10 min of incubation at 37°C, the reaction was terminated by addition of 0.9 ml of 50 mM sodium acetate buffer (pH 4.0) and boiling the tubes for 3 min. The formed cAMP was determined by radioimmunoassay the same as the described above. The adenylate cyclase activity was expressed as pmoles/mg protein/10 min.

Assay of guanylate cyclase

The assay medium contained 50 mM Tris-HCl (pH 7.6), 10 mM theophylline, 15 mM creatine phosphate, 20 μg creatine phosphokinase (100-150 units/mg), 4 mM $MnCl_2$, 1 mM GTP and enzyme preparation (40-60 μg protein) in a final volume of 100 μl. The assay of guanylate cyclase activity was performed in the same way as described for assay of adenylate cyclase. The guanylate cyclase activity was expressed as pmoles cGMP/mg protein/10 min.

Statistics

All values were expressed as the mean ± S.E.. The difference between means was assessed for significance by Student's *t* test. Values of $p<0.05$ were taken as statistically significant.

RESULTS

Clearance results

After hydrochlorothiazide administration, maximal diuretic and saluretic effect was obtained during the second collection period (10-20 min period). During this period, urin flow and urinary sodium, chloride, and potassium excretion were significantly increased. GFR and RPF were slightly

Table 1. Effect of hydrochlorothiazide on renal function in rabbits

Drug	U_{vol} (ml/min)	$U_{Na}{}^+V$ (μEq/min)	$U_K{}^+V$ (μEq/min)	$U_{Cl}{}^-V$ (μEq/min)	C_{osm} (ml/min)	C_{In} (ml/min)	C_{PAH} (ml/min)
Control	0.265 ± 0.018	64.05 ± 1.23	5.890 ± 0.261	64.85 ± 1.73	0.659 ± 0.025	7.158 ± 0.517	31.16 ± 2.32
HCTZ	0.678 ± 0.071[a]	145.72 ± 8.89[a]	11.956 ± 0.829[a]	131.69 ± 9.92[a]	1.195 ± 0.072[a]	6.913 ± 0.244	28.95 ± 1.30

Mean ± S.E. a: $p<0.001$ numbler of rabbits = 8
U_{vol} : urine volume
$U_{Na}{}^+V$: sodium excretion rate
$U_K{}^+V$: potassium excretion rate
$U_{Cl}{}^-V$: chloride excretion rate
C_{osm} : osmolar clearance
C_{In} : inulin clearance
C_{PAH} : PAH clearance
HCTZ : hydrochlorothiazide 5 mg/kg, i.v.

Table 2. Effect of hydrochlorothiazide on the renal tissue level of cAMP in rats

Time (min)	cAMP level (p.moles/mg protein)	
	Saline	HCTZ
0	6.003 ± 0.219	
	(7)	
5	6.032 ± 0.218	5.242 ± 0.229[a]
	(10)	(10)
15	6.021 ± 0.215	4.794 ± 0.240[b]
	(10)	(10)
60	6,016 ± 0.223	5.847 ± 0.228
	(10)	(10)

Mean ± S.E. a:$p<0.05$ b:$p<0.01$
( ) = number of rats
HCTZ: hydrochlorothiazide 5 mg/kg, i.v.

Table 3. Effect of hydrochlorothiazide on the renal tissue level of cGMP in rats

Time (min)	cGMP level (pmoles/mg protein)	
	Saline	HCTZ
0	0.451 ± 0.019	
	(7)	
5	0.445 ± 0.022	0.460 ± 0.024
	(10)	(10)
15	0.456 ± 0.021	0.485 ± 0.026
	(10)	(10)
60	0.457 ± 0.024	0.464 ± 0.023
	(10)	(10)

Mean ± S.E. ( ) = number of rats
HCTZ: hydrochlorothiazide 5 mg/kg, i.v.

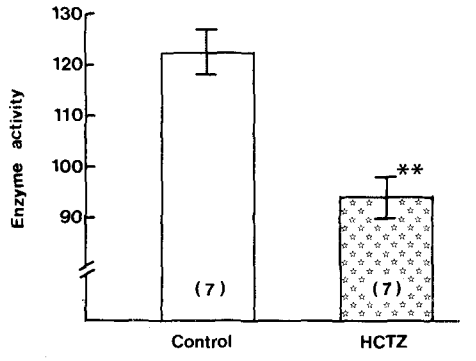

Fig. 1. Effect of hydrochlorothiazide on rat kidney adenylate cyclase activity in vitro. Adenylate cyclase activity was expressed as pmoles cAMP/mg protein/10 min.
Mean ± S.E. **$p<0.01$
HCTZ: hydrochlorothiazide 5×10^{-4}M
( ) = number of experiments

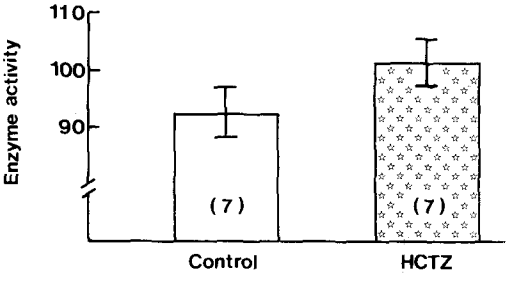

Fig. 2. Effect of hydrochlorothiazide on rat kidney guanylate cyclase activity in vitro. Guanylate cyclase activity was expressed as pmoles cGMP/mg protein/10 min.
Mean ± S.E.
HCTZ: hydrochlorothiazide 5×10^{-4}M
( ) = number of experiments

decreased but these changes were not significant (Table 1). Urine flow and urinary electrolytes excretion returned to the control levels 60 min after injection of hydrochlorothiazide.

Effect of hydrochlorothiazide on the renal tissue level of cyclic nucleotides

5, 15 and 60 min after injection of isotonic saline (0.5 ml/kg), the cAMP levels of renal tissue were 6.031 ± 0.218, 6.02 ± 0.215 and 6.016 ± 0.223 pmoles/mg protein, respectively (Table 2), and the cGMP levels of renal tissue were 0.445 ± 0.022, 0456 ± 0.021 and 0.457 ± 0.024 pmoles/mg protein, respectively (Table 3). These levels were not different from the basal levels of the renal tissue cAMP (6.003 ± 0.219 pmoles/mg protein) and cGMP (0.451 ± 0.019 pmolels/mg protein).

The cAMP level of renal tissue was decreased at 5 and 15 min after injection of hydrochlorothiazide, but the cAMP level was not different from control at 60 min after injection (Table 2). The cGMP level of renal tissue was not affected by hydrochlorothiazide (Table 3).

Effect of hydrochlorothiazide on the renal adenylate cyclase and guanylate cyclase activity

Basal activity of renal adenylate cyclase was 122.49 ± 4.02 pmoles cAMP/mg protein/10 min. Hydrochlorothiazide 5×10^{-4}M decreased the renal adenylate cyclase activity (Fig. 1.)

Basal activity of renal guanylate cyclase was 93.34±3.56 pmoles cGMP/mg protein/10. The renal guanylate cyclase activity was not significantly affected by hydrochlorothiazide (Fig. 2).

DISCUSSION

The diuretic effect of thiazides is due to the inhibition of the sodium reabsorption in the renal tubules. Although the thiazides have an inhibitory effect on active transport in the proximal tubule and the collecting duct (Beyer, 1958; Maren, 1967; Wilson *et al.*, 1983), they have a major site of action in the distal convoluted tubule (Fernandez & Puschett, 1973; Edwards *et al.*, 1973; Kunau *et al.*, 1975; Constanzo & Windhager, 1978; Hansen *et al.*, 1981; Velazquez & Wright, 1983). However, the mechanism of sodium reabsorption in the distal convoluted tubule is obscure, and, thus, so is the detailed mechanism of action of the thiazides. A $Na^+/2Cl^-/K^+$ cotransport system which was found in the thick ascending limb of Henles' loop (Greger & Schlatter, 1981; Greger *et al.*, 1983; Greger & Schlatter, 1983; Steven & Andreoli, 1984) and the distal nephron of amphiuma kidney (Oberleithner *et al.*, 1983a; Oberleithner *et al.*, 1983b). In addition, there does not appear to be any mineralocorticoid-dependent Na^+ transport system in the distal convoluted tubule (Berger & Warnock, 1985). Recently, Stokes (1984) demonstrated a thiazide-sensitive electrically neutral NaCl transport system in the urinary bladder of the winter flounder. This system was inhibited by thiazide analogs, but it was not affected by loop diuretics, amiloride, carbonic anhydrase inhibitors, and 4,4'-diisothiocyano-2,2'-disulfonic stilbene, an inhibitor of anion exchange. He proposed that this transport system probably exist in the distal convoluted tubule or the medullary collecting tubule. However, it has not been determined whether this transport system is present in the renal tubule of the mammalian kidney, and the mechanism through which the thiazides interact with the NaCl transport system is unclear.

There is a close corelation between the cyclic nucleotides and the renal electrolytes reabsorption. Steven and Andreoli (1984) suggested that in the medullary thick ascending limb of Henle's loop cells cAMP increases the number of apical potassium channels and apical $Na^+/2Cl^-/K^+$ cotransporter molecules, and Schlatter and Greger (1985) proposed that cAMP increased the basolateral Cl^--conductance in the medullary thick ascending limb of Henle's loop of the mouse. cAMP, also, inhibited a carbonic anhydrase (Beck *et al.*, 1975; McKinney & Myers, 1980). Osswald (1974) demonstrated that cGMP injected into the thoracic aorta of rats induced an increase of the renal electrolyte excretion. Lee and Cho (1983), and Lee *et al.*, (1985) proposed that furosemide action may be related to the renal tissue level of cGMP. In this study, hydrochlorothiazide decreased the tissue level of cAMP in the rat kidney. Although hydrochlorothiazide has an inhibitory effect on the carbonic anhydrase, it is supposed that the decrease of cAMP level is largely attributed to the effect on the distal convoluted tubule because acetazolamide, a carbonic anhydrase inhibitor, increase cAMP production by stimulating the renal adenylate cyclase (Rodriguez *et al.*, 1974; Jacobsen & Kokko, 1976). But, as hydrochlorothiazide has an inhibitory effect on chloride reabsorption in the medually collecting duct (Wilson *et al.*, 1983), the possibility that the decrease of cAMP level is at least partially due to this effect can not be rejected. Moore (1968) and Senft *et al.*, (1968) demonstrated the inhibitory effect of hydrochlorothiazide on cAMP phosphodiesterase, but in this study it, also, inhibited the renal adenylate cyclase. Therefore, the decrease of cAMP level is due to the inhibition of adenylate cyclase. The renal tissue level of cGMP and guanylalte cyclase activity was not affected by hydrochlorothiazide. Thus, cGMP is not related to the renal effect of hydrochlorothiazide. Further studies on the relation between cAMP and electrolyte reabsorption in distal tubule is necessary.

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＝국문초록＝

Hydrochlorothiazide가 신장의 Cyclic Nucleotides 함량에 미치는 영향

가톨릭의과대학 약리학교실

이석용, 고택립, 이우영, 이상복, 조규철

hydrochlorothiazide의 이뇨작용과 cyclic nucleotides와의 관계를 알아보기 위해 신조직내 cyclic nucleotides 함량과 adenylate cyclase 및 guanylate cyclase 활성에 대한 hydrochlorothiazide의 영향을 관찰하였다. hydrochlorothiazide를 정맥내 투여시 약물투여 후 10분과 20분 사이에서 이뇨작용이 가장 강하게 나타났으며 60분 경과시는 이뇨작용이 소실되었다. 신조직내 cAMP 함량은 약물투여 후 5분과 15분에 유의하게 감소되었으며 60분 경과시는 대조군과 차이가 없었다. 신조직내 cGMP 함량은 hydrochlorothiazide에 의해 영향받지 않았다. 신조직의 adenylate cyclase는 hydrochlorothiazide에 의해 활성이 억제되었으며 guanylate cyclase는 영향받지 않았다.

이상의 결과는 hydrochlorothiazide의 이뇨작용에 cAMP가 어떤 관련성을 가질 것을 시사하며 cGMP는 관련성이 없는 것으로 사료된다.