Studies of the Elevated Extracellular Concentration of Cyclic AMP in Uremic Man

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ABSTRACT This study was designed to elucidate the mechanism of elevation of plasma cyclic AMP in uremic man. Plasma cyclic AMP was measured in 15 normal subjects and in 18 patients with severe renal failure. In some members from both groups the kinetic parameters of the metabolism of extracellular cyclic AMP were measured.

Plasma cyclic AMP was elevated from 23 nM in control subjects to 59 nM in uremic patients, regardless of the presence or absence of the kidneys or parathyroid glands. A single pass of uremic blood through a Kiil hemodialyzer decreased plasma cyclic AMP from 58 to 30 nM. The clearance of cyclic AMP by the dialyzer correlated directly with the blood flow passing through the machine. Hemodialysis for 6 h decreased plasma cyclic AMP levels in the systemic circulation by only 12%. Studies with tritiated cyclic AMP revealed a plasma clearance rate of 624 ml/min in normal subjects and of 344 ml/min in patients with uremia. Such a large decrease in plasma clearance rate cannot be explained by a failure of urinary excretion of cyclic AMP and suggests impairment of "metabolic clearance." In addition, the "plasma production rate" of cyclic AMP was 65% higher in patients with renal failure than in normal subjects.

It is concluded that the elevation of plasma cyclic AMP in uremic man is due to a combination of: (*a*) lack of urinary excretion, (*b*) decreased metabolic clearance, and (*c*) increased production of plasma cyclic AMP.

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INTRODUCTION

Previous studies have shown plasma levels of cyclic AMP (adenosine 3′,5′-monophosphate) to be abnormally high in anephric man (1), and urinary excretion of the cyclic nucleotide has been observed to be decreased in patients with azotemia (2). Broadus, Kaminsky, Hardman, Sutherland, and Liddle demonstrated that urinary excretion of cyclic AMP accounts for about 15–20% of its total disposal in normal man (3). From this it was inferred that the absence of urinary excretion could not explain the magnitude of the increase in plasma cyclic AMP levels in anephric man. In the present study we have attempted to ascertain whether the increased plasma cyclic AMP found in uremia is due to increased cyclic AMP formation, decreased removal, or both.

METHODS

Materials

Cyclic AMP was purchased from Schwartz Bio-Research Inc. (Orangeburg, N. Y.), [^{3}H]cyclic AMP (33.2 Ci/mmol) from New England Nuclear (Boston, Mass.), and Dowex AG 50W-X8 (hydrogen form, 100–200 mesh) from Bio-Rad Laboratories (Richmond, Calif.). All other reagents were of analytical grade. For human use the tritium-labeled cyclic AMP was first purified by column chromatography as described below. The eluates were sterilized by double passage through a 0.45-μm Millipore filter. Aliquots of the stock solution were transferred with sterile technique to vials of apyrogenic saline solution (for multiple use). Immediately before use, the solution for injection was neutralized by addition of an appropriate quantity of sodium bicarbonate so that the final pH was 7.4. Aliquots of these solutions were repeatedly tested by an independent laboratory and found to be free of microorganisms and pyrogens

Subjects and procedures

15 healthy adult volunteers and 18 patients with advanced renal failure were examined. The mean ages of the two

groups were not significantly different (35±7[SD] and 41 ±13 in controls and patients, respectively). There was similar representation of sex and race in both groups.

Blood samples. Blood samples for plasma levels of cyclic AMP were obtained at random from both groups during casual daily activity. In patients undergoing chronic hemodialysis, blood samples were drawn immediately before dialysis (unless otherwise stated). Blood samples were drawn into chilled 10-ml glass tubes containing 15 mg of EDTA to inhibit destruction of cyclic AMP and were immediately centrifuged at 4°C. 5 ml of plasma were then separated and frozen at −70°C for later assay. In the presence of EDTA, cyclic AMP in the samples was stable in the unfrozen state for at least 24 h. This was ascertained by incubating samples at room temperature and determining the recovery of [^{3}H]-cyclic AMP. The recovery of cyclic AMP from samples incubated in the presence of EDTA was quantitative, whereas that from samples incubated in the absence of EDTA was only about 5% of the amount added.

Study of clearance of cyclic AMP by hemodialyzer. Six patients with renal failure and undergoing chronic hemodialysis using the standard Kiil dialyzer were studied during the dialysis procedure after an initial period of stabilization (4). Simultaneous samples from "arterial" and "venous" lines of the dialyzer were obtained during stable blood flow rates which were varied for each patient from 120 to 200 ml/min. Similar samples were also obtained for blood urea nitrogen (BUN) [1] and creatinine measurements. The clearance of each substance was calculated from the formula:

$$C_p = Q_b\,(1 - \text{PCV}/100)\,([B_i] - [B_o])/[B_i],$$

where C_p = plasma clearance (milliliters/minute), Q_b = blood flow through the dialyzer (milliliters/minute), $[B_i]$ = concentration of the substance in the plasma flowing into the machine, $[B_o]$ = concentration of the substance in the plasma flowing from the machine, and PCV = packed cell volume. The elimination rate achieved by the hemodialysis was calculated as the product of the clearance term and the plasma level of cyclic AMP, urea, or creatinine (i.e., $C_p \times B_i$).

Study of kinetic parameters of plasma cyclic AMP. This study was performed with a single, rapid intravenous injection of [^{3}H]cyclic AMP as described by Broadus et al. (3). Separate aliquots were counted for "total radioactivity" and purified as described below for determination of cyclic AMP. The procedural loss of the tritiated cyclic AMP was evaluated by monitoring the recovery of added nonradioactive cyclic AMP as estimated by absorbance at 258 nm. Three additional samples were drawn during each study for the measurement of endogenous levels of cyclic AMP and handled as described below. The amount of [^{3}H]cyclic AMP injected did not detectably alter the level of total cyclic AMP in plasma. Finally, another three samples were drawn into tubes containing heparin and then treated with cyclic nucleotide phosphodiesterase as described later. These latter samples did not show any detectable radioactivity after the purification used in this procedure.

All mathematical methods used are those generally described for hormonal kinetic studies (5–9) as adapted for the study of cyclic nucleotides by Broadus et al. (3, 10).

Measurement of cyclic AMP. Plasma for measurement of endogenous levels of cyclic AMP was quickly thawed, and 3 nCi of [^{3}H]cyclic AMP was added to 5 ml of plasma. Next, 1 ml of 0.8 M zinc acetate and 1 ml of 0.8 M sodium carbonate were added, and after completion of precipitation, the samples were centrifuged at 18,000 *g* for 10 min at 4°C. The supernatant fluid was transferred to a tube containing 1 ml of 60% trichloroacetic acid and centrifuged again under the same conditions (after standing for 1 h in a 55°C water bath). The supernatant fluid was then applied to a 0.7 × 30-cm column of Dowex 50W equilibrated with 0.1 N HCl. The cyclic AMP fraction was collected between 40–85 ml of eluent (with 0.1 N HCl) and lyophilized. Samples were then resuspended in 1 ml of sodium acetate buffer (pH 4), and 100 μl was used for measurement of recovery while 50, 25, and 12.5 μl were assayed for cyclic AMP as described by Gilman (11).

The specificity of the assay was verified by measurements of the cyclic AMP content of representative samples (all samples of endogenous cyclic AMP involved in the kinetic studies) after incubation for 16 h with 3′,5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.1) (12). 5 U of the enzyme and $MgCl_2$ to final concentration of 2 mM were added to 2.5 ml of plasma. By use of the previously described purification procedure, phosphodiesterase-treated samples were found to contain a "cyclic AMP-like" blank equivalent to about 5% of the value determined before treatment with phosphodiesterase; this was subtracted from values of samples involved in kinetic studies.

The intra-assay coefficient of variation of the three different dilutions was 14.8±1.2%. The mean of these three dilutions is presented. The coefficient of variation of routinely reassayed, pooled plasma measured with each run was 6.4% (for 14 measurements). The mean recovery of [^{3}H]cyclic AMP added to a sample of plasma was 52.2± 0.9% for 96 samples involved in the present study. Every sample was corrected for its recovery. All values are means ±SEM.

All other biochemical determinations (blood calcium, phosphate, creatinine, and urea nitrogen) were performed by routine, automated procedures (13–15).

RESULTS

Plasma levels of cyclic AMP. Individual clinical data and the endogenous plasma levels of cyclic AMP in 18 patients with advanced renal failure are shown in Table I.

Plasma levels of cyclic AMP in patients with renal failure were more than twice as high as in control subjects. Plasma levels in control subjects were similar to published values (3, 16). The elevated cyclic AMP levels were independent of the presence or absence of the kidneys or of parathyroid glands (Fig. 1). Similar levels were observed in six patients with bilateral nephrectomy and in four patients with parathyroidectomy requiring calcium and vitamin D administration. The values in the operated patients were obtained several weeks to years postoperatively.

Although several patients were receiving medications (vitamins, antihypertensives, cardiac glycosides, and anticoagulants), ancillary studies in uremic human beings and dogs not receiving medications indicated that the high levels of plasma cyclic AMP were characteristic of the uremic state per se.

[1] *Abbreviation used in this paper:* BUN, blood urea nitrogen.

TABLE I

Clinical Data and Plasma Cyclic AMP in Chronic Uremia

Subject	Age	Sex	BUN*	Calcium*	Phosphate*	PTX‡	NPX§	Dialysis‖	cAMP
				mg/100 ml					*nM*
E. B.	20	F	83	9.5	5.5	—	+	+	66.6
W. D.	20	M	50	9.3	4.6	+	—	+	69.6
G. W.	22	M	94	8.8	5.5	+	—	—	44.5
R. D.	24	M	85	10.8	3.1	—	+	+	43.7
S. W.	32	F	120	9.3	5.6	—	+	+	54.4
R. G.	34	M	139	7.6	4.8	+	—	+	52.8
G. C.	39	F	103	8.0	8.4	—	—	—	54.7
P. J.	43	M	50	10.5	3.9	—	+	+	55.8
W. M.	44	M	91	8.0	6.8	—	—	+	48.5
W. L.	46	M	44	9.8	3.4	—	—	+	35.8
T. L.	47	M	125	9.9	5.4	—	+	+	75.6
P. W.	49	M	150	9.4	6.6	—	+	+	99.4
W. R.	49	M	126	7.5	3.1	—	—	+	59.3
L. M.	51	F	74	7.8	6.3	+	—	+	64.3
G. J.	51	F	65	10.5	7.8	—	—	+	51.2
H. B.	53	F	69	9.4	4.4	—	—	+	58.9
A. W.	60	M	130	8.9	1.7	—	—	+	76.0
B. J.	67	M	100	8.8	5.7	—	—	+	56.9

* Serum measurements.

‡ PTX, subjects with parathyroidectomy.

§ NPX, subjects with bilateral nephrectomy.

‖ Dialysis, subjects on chronic hemodialysis.

The effect of successful renal transplantation on the plasma level of cyclic AMP was evaluated in one patient. The plasma cyclic AMP was 67 nM after the bilateral nephrectomy, and it fell to 32 nM 2 wk after the transplantation and rose to 47 during an episode of graft rejection.

Effect of hemodialysis on cyclic AMP. 16 of the 18 subjects with renal failure in the present study were undergoing hemodialysis several times a week for 6–8 h with the Kiil dialyzer. Plasma levels in the two patients not yet having been subjected to dialysis were similar to those of the chronically dialyzed patients. Samples of peripheral blood were drawn from four different patients immediately at the beginning and again at the end of the dialysis sessions; cyclic AMP levels in these samples from the systemic circulation declined only slightly, from a mean of 59.0±7.8 to 51.7±6.0 nM.

The efficacy of hemodialysis on cyclic AMP was studied by simultaneous sampling from arterial and venous lines of the dialyzer as described in Methods. Over the range of blood flow used (120-200 ml/min), clearances of BUN, creatinine, and cyclic AMP were dependent on the blood flow passing through the dialyzer (the correlation coefficient was highly significant, $r = 0.92$, 0.93, and 0.97, respectively). It appears (Table II) that the clearance rates of the substances were inversely related to their molecular weights.

Kinetic studies of cyclic AMP. Fig. 2 demonstrates the differences in the rates of disappearance of injected [^{3}H]cyclic AMP from the circulation in control subjects and in uremic patients. A reasonable fit to straight lines was obtained by resolving the data from each individual into two exponential components (α = rapid

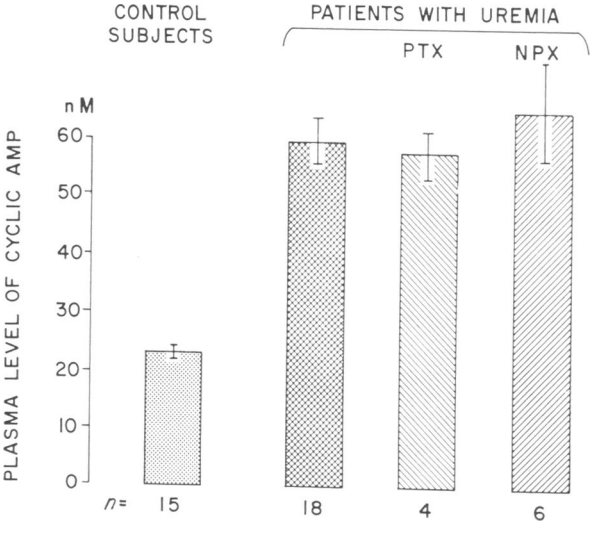

FIGURE 1 Plasma levels of cyclic AMP in 15 control subjects and 18 patients with chronic renal failure. Four of the uremic patients had undergone parathyroidectomy (PTX) and six bilateral nephrectomy (NPX).

TABLE II

*Dialysis Parameters of BUN, Creatinine, and Cyclic AMP**

	BUN	Creatinine	Cyclic AMP
(Mol wt)	(60)	(113)	(329)
Input‡	54±3 mg/100 ml	9.2±0.4 mg/100 ml	58±2 nmol/liter
Output‡	13±1 mg/100 ml	3.7±0.2 mg/100 ml	30±2 nmol/liter
Clearances§	96±5 ml/min	74±4 ml/min	60±5 ml/min
Elimination rate	52±4 mg/min	6.8±0.3 mg/min	3.3±0.4 nmol/min

* Mean (±SEM) of 12 measurements, mean blood flow of 155 ml/min, mean packed cell volume = 16.7%.

‡ Input and output values are the concentrations of substances in plasma of blood flowing into and out of the hemodialyzing machine, respectively.

§ Clearance of given substance by dialyzing machine.

phase and β = slow phase) by standard techniques (9). After the initial distributive phase (first 20 min) during which the plasma concentrations of [^{3}H]cyclic AMP in uremic subjects fell below those in normal subjects, the concentrations of plasma [^{3}H]cyclic AMP declined at a much slower rate in uremic than in normal subjects.

The details of the kinetic parameters in individual studies are shown in Table III. A significant difference is observed in the β, slow phase, the so-called elimination phase ($P < 0.005$ by t test). The plasma level of endogenous cyclic AMP, measured three times during the study, was stable (mean values are shown in Table III). The area under the curve of disappearance of injected [^{3}H]cyclic AMP was appreciably larger in the uremic patients than that in the control subjects (Fig. 2). This permits the conclusion that the plasma clearance rate was significantly lower in uremic patients ($P < 0.001$) (Table III). In addition, the production rate of plasma cyclic AMP in uremic patients was computed to be significantly higher ($P < 0.001$); thus, their high plasma levels could not be accounted for on the basis of decreased plasma clearance only. The two independent measurements–plasma clearance rate (estimated from dpm of [^{3}H]cyclic AMP) and the concentration of endogenous cyclic AMP (measured by protein binding)–showed a highly significant negative correlation ($r = -0.92$, $P < 0.01$).

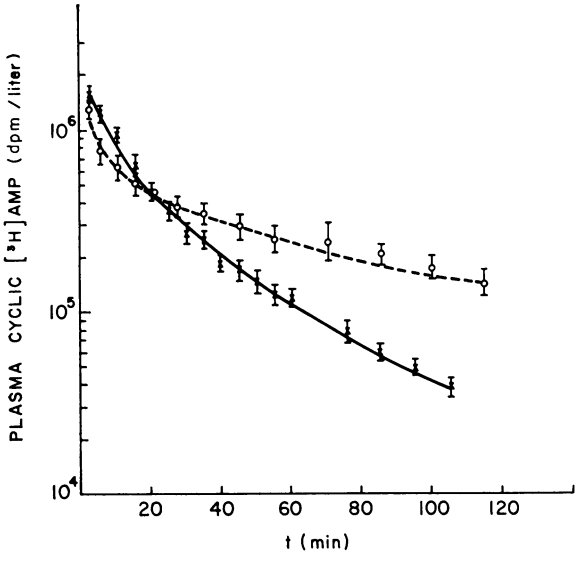

FIGURE 2 Plasma [^{3}H]cyclic AMP disappearance curves after a single intravenous injection in control subjects and in patients with chronic renal failure. Plasma [^{3}H]cyclic AMP was purified as described in Methods. O---O represents the means of four uremic patients and ×——× the means of five control subjects. Vertical bars represent standard errors of the mean.

Finally, the reversible part of the tracer flux between the compartments was calculated (3, 6). The advantage of this calculation is that it is independent of assumptions about the number and nature of the pools exchanging with the circulating compound (6). It appears that the percentage of reversible tracer flux is significantly increased in uremia ($P < 0.05$) (Table III).

The plasma levels of cyclic AMP in three of the four uremic subjects included in the kinetic studies were coincidentally above the mean level of all patients. However, the results of the kinetic studies were similar in these three and the fourth subject, R. G., whose level of plasma cyclic AMP was slightly below the mean of the entire group of patients.

DISCUSSION

Chronic renal failure is accompanied by a wide spectrum of disturbances in the transport (17, 18) and metabolism of many substances including several hormones (19–26). Impaired renal function has been shown to result in abnormal metabolism of hormones not only via decreased glomerular filtration but also via decreased destruction of the substance by renal parenchyma (22–26).

TABLE III

Kinetic Parameters of Extracellular Cyclic AMP

Subject	Dose	Plasma [^{3}H]cAMP*				$t_{\frac{1}{2}}$		Plasma clearance	Plasma cAMP	Plasma production rate	Initial volume of distribution	Reversible tracer flux ($1-f$)
		A	B	α	β	$0.693/\alpha$	$0.693/\beta$					
	μCi (nCi/kg of body wt)					*min*	*min*	*ml/min*	*nM*	*nmol/min*	*liters*	%
Control subjects												
H. B.‡	13.58 (169)	1.824	0.781	0.1145	0.0290	6.05	23.90	697	23.7	16.5	11.57	31.6
T. F.‡	12.12 (133)	2.350	0.609	0.1048	0.0227	6.61	30.53	542	24.2	13.1	9.09	31.4
J. C.‡	10.45 (143)	2.046	0.368	0.1288	0.0216	5.38	32.08	692	15.0	10.4	9.43	34.8
L. Y.	10.10 (131)	1.237	0.374	0.0762	0.0219	9.09	31.52	673	20.6	13.8	13.90	23.8
B. C.	11.47 (144)	2.264	0.579	0.0806	0.0275	8.59	25.19	518	27.9	14.4	8.95	17.2
Mean						7.14	28.64	624	22.3	13.6	10.59	27.8
±SEM						0.72	1.70	39	2.1	1.0	0.95	3.2
Patients with chronic renal failure												
R. G.	9.59 (144)	1.403	0.513	0.0733	0.0136	9.45	50.81	375	52.8	19.8	11.11	41.1
A. W.	10.48 (187)	1.571	0.598	0.0694	0.0133	9.97	52.17	343	76.0	26.1	10.72	40.7
T. L.§	8.64 (113)	0.811	0.366	0.1061	0.0082	6.53	84.41	363	75.8	27.5	16.29	77.4
P. W.§	11.25 (149)	1.548	0.731	0.0619	0.0119	11.19	58.06	296	99.4	29.4	10.95	41.1
Mean						9.28	61.36	344	76.0	25.7	12.26	50.1
±SEM						1.98	7.84	17	9.5	2.1	1.34	9.1

* The concentration of [^{3}H]cyclic AMP $= Ae^{-\alpha t} + Be^{-\beta t}$, where A and B are the intercepts of "fast" and "slow" components (in dpm × 10^6/liter), α and β are the fractional rates (per minute), and t is the time (in minutes).

‡ Kinetic studies in these three subjects have been previously reported by Broadus et al. (3) (reproduced here by permission of authors and publisher).

§ Patients with bilateral nephrectomy.

Broadus et al. demonstrated that urinary excretion in normal man accounts for only about 15–20% of total cyclic AMP disposal (3). Thus, the complete absence of urine formation alone would increase the plasma level only from about 22 to 27 nM in the new steady state.[2] In the present study, the plasma clearance of cyclic AMP was, on the average, 270 ml/min less in uremic patients than in normal subjects, a difference that is not fully accounted for by the mere lack of normal glomerular filtration. It is concluded on this basis that the metabolic clearance of cyclic AMP in uremia is decreased. Although Schneider and Jutzler (27) have reported that the elevated plasma levels of cyclic AMP in uremia were correlated with the reduction in glomerular filtration rate, a number of other observations support the finding of the present study that a reduction in glomerular filtration rate cannot entirely account for the degree of elevation of plasma cyclic AMP. Recently, Wehmann, Blonde, and Steiner (28, 29), using infusions of tritiated cyclic nucleotides in dogs, observed that urinary excretion accounted for only about two-thirds of the total kidney clearance of cyclic AMP, extraction of the nucleotide by renal parenchymal tissue apparently accounting for the remainder. A significant role of renal extraction, in contrast to excretion, was also demonstrated by the experiments of Coulson, Roch-Ramel, and Bowman, who studied the disappearance of [^{3}H]cyclic AMP from plasma before and after nephrectomy in rats (30) and by those of Coulson and Bowman (31), who studied the handling of extracellular cyclic AMP by the perfused rat kidney. The extraction of cyclic AMP by kidney parenchyma appears to be dependent upon a transport system that can be blocked by probenicid (30, 31). Thus, the kidney appears to participate in the metabolic clearance of plasma cyclic AMP in addition to the urinary excretion of the nucleotide.

[2] In the steady state, total disposal rate = concentration of cyclic AMP × plasma clearance = plasma production rate; i.e., concentration of cyclic AMP = plasma production rate × (metabolic clearance + kidney clearance)$^{-1}$. By substituting 0.120 liter/min for kidney clearance (3) and using other normal values from Table III, it can be seen that if metabolic clearance and production were unchanged, the absence of kidney clearance would result in a concentration of cyclic AMP equal to: $13.6 \times (0.624 - 0.120)^{-1} = 27$ nM.

Furthermore, even the decrease in the plasma clearance of cyclic AMP does not entirely explain the elevated plasma level of the nucleotide in uremia. An increase in production rate appears to be another contributing factor (Table III). The increase of the "plasma production rate" could be secondary to increased activity of adenylate cyclase (conceivable because of increased levels of several hormones) and/or to decreased activity of phosphodiesterase. Alternatively, the increased "plasma production rate" and decreased metabolic clearance could be related to abnormal transport of cyclic AMP between compartments, since an increased percentage of "reversible flux" of extracellular cyclic AMP was found in uremic patients (Table III). At present, it is not possible to identify the anatomical "compartments" involved in the flux of cyclic AMP into and out of the plasma. Theoretically, if metabolic clearance of plasma cyclic AMP involves saturable processes, an increased production rate could contribute to a reduced metabolic clearance rate by elevating plasma cyclic AMP to near-saturating concentrations.

Several abnormalities in uremia including decreased platelet adhesiveness and abnormal sodium transport have been attributed to the presence of unidentified "toxins" in plasma (32–34). Whether or not such factors might influence the metabolism of cyclic AMP is unknown. Interestingly, cyclic AMP itself has been reported to be involved in the physiological regulation of both platelet adhesiveness (35, 36) and sodium transport (37–39). Although this study has not delineated a role for cyclic AMP in the pathophysiology of any abnormalities in uremia, the fact that the metabolism of *extracellular* cyclic AMP is abnormal in uremia should encourage studies of the nucleotide's *intracellular* metabolism and possible involvement in other pathological aspects of this disease.

Note added in proof. We have recently also measured cyclic GMP in uremic and control subjects included in this present study. Cyclic GMP levels were 4.2±SEM 0.7 nM ($n = 9$) in control subjects and 19.5±2.9 nM ($n = 5$) in uremics. A single passage of blood through a dialyzing machine decreased cyclic GMP levels in uremics to 12.9±1.2 nM.

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