In Vitro Stimulation of Renin Production by Epinephrine, Norepinephrine, and Cyclic AMP* (33647)

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Although the physiologic regulation of renin production by the kidney has been the subject of numerous studies, it remains incompletely understood. There is evidence that conditions that lower the perfusion pressure of blood in the renal artery stimulate renin secretion (1, 2). Many of these conditions are associated with contraction or redistribution of blood volume in such a way that reflex activation of the sympathetic nervous system occurs, and it has been suggested that the sympathetic nervous system plays a role in mediating the renin-secretory responses to such stimuli as upright posture and sodium depletion (3). It has been reported that direct stimulation of the renal nerves and the infusion of catecholamines are both effective stimuli to renin production (3–5). From published studies, it is not clear whether catecholamines exert their action by constricting the afferent arterioles or by a direct stimulatory effect on the renin-producing cells.

The fact that a noncatecholamine pressor agent, angiotensin II, causes arteriolar constriction while causing a decrease in renin production suggests that renal hemodynamic factors per se are not all-important in regulating renin secretion (5, 6).

In order to determine whether catecholamines might influence the production of renin by a direct chemical action on the renal cell, we developed a renal cell suspension system which is free of hemodynamic or neurogenic influences. In this suspension we have studied the effects of epinephrine, norepinephrine, and adenosine-3′,5′-cyclic monophosphoric acid (cyclic AMP) on the production of renin. Since the method we employed does not provide information concerning the rate of synthesis and the rate of destruction of renin as independent processes, only their algebraic sum is known, i.e., "net production of renin."

Materials and Methods. Materials. Medium. To each 100 ml of sterile MEN Eagle, Spinner modified medium without glutamine (Baltimore Biological Laboratory, Baltimore, Maryland), 1.3 ml of 200 m*M* L-glutamine sterile solution (Robbin Laboratories, Inc., Chapel Hill, North Carolina) was added (7), and the resulting solution was used as the medium in all experiments. The pH was 7.2.

Collagenase. A product of Nutritional Biochemicals Corp., Cleveland, Ohio.

Adenosine-3′,5′-cyclic monophosphoric acid. Cyclic AMP, product of SIGMA Chemical Company, St. Louis, Missouri.

Methods. Preparation of the renal cell suspension. Dog kidneys were processed by the technique of Burg and Orloff (8). Without special dietary preparation, young mongrel dogs weighing 12–20 kg were anesthetized with pentobarbital. Through an abdominal incision both kidneys were excised and immediately placed in ice. A needle was tied in place in each renal artery, and the kidney was perfused with physiological saline followed by medium until the fluid coming out through the renal vein was clear. The renal vein was then occluded with ligatures and a freshly prepared solution of 0.3% collagenase in medium was forcibly injected into the kidney via the renal artery until fluid escaped through the surface of the kidney. The cortex was removed and minced with scissors to pieces less than 3 mm in size. These fragments were washed once with 5 ml of 0.3% collagenase solution in medium and then placed in 50 ml of the same solution for digestion. The digestion mixture was slowly

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stirred for 1 hr with a magnetic stirrer under an atmosphere of 95% oxygen and 5% carbon dioxide. The mixture was diluted to 100 ml with medium and then filtered in the cold through a double layer of 20 × 12 mesh cotton gauze. The filtrate was then centrifuged in the cold for 3 min at 50*g*, and the supernatant was discarded. The sediment was washed with medium and centrifuged as above, three times, in order to remove collagenase. Finally, the cells were suspended in medium and within 30 min were used in the experimental incubations as described below. Each suspension, examined microscopically, consisted of intact cells which stained with methylene blue. Some tubular fragments were found, but separated glomeruli were not identified.

Experimental incubations. In each experiment, 5-ml aliquots of the well-mixed suspension were added to each flask. Cell breakage during transfer was minimized by using pipettes with wide tips. In each experiment, one or more flasks were frozen at the beginning of the incubation period to serve as "nonincubated controls." Other flasks were incubated in medium alone to serve as "incubated controls." As experimental variables, epinephrine, norepinephrine, or cyclic AMP were added to other incubated flasks. Experiments were also performed in which cycloheximide was added to alternate flasks. Most samples were run in duplicate. All incubations were carried out for 1 hr in a 37° water bath with slow constant shaking under an atmosphere of 95% oxygen and 5% carbon dioxide. At the end of the incubation period, the suspensions (including the supernate) were homogenized in a Potter-Elvejhem tube with a Teflon pestle, diluted with medium, and immediately transferred to plastic vials containing 0.1 ml of 1 *N* hydrochloric acid (9) and stored at —20° until the time of renin determination.

Preparation of renin substrate. After bilateral nephrectomy, each dog was allowed to remain under anesthesia for at least 4 hr, and then blood was drawn from the aorta into tubes containing ammonium EDTA as anticoagulant. Without delay, the blood was centrifuged in the cold, and the plasma was stored at —20° until use. This plasma was then used as an autologous substrate for the measurement of renin activity in each of the cell suspensions.

Renin measurement. Homogenized aliquots of each flask of cell suspension were added to 10 ml of the above renin substrate, and angiotensin was generated and trapped during a 3-hr incubation period [modification (9) of the method of Boucher and co-workers (10)]. "Renin activity" values are reported as micrograms (μg) of generated angiotensin. The adequacy of substrate in the plasma was checked in each experiment by using graded quantities of cell suspension and showing that the quantity of angiotensin generated was proportional to the quantity of cell suspension. In all experiments, the renin activity in the substrate plasma prior to addition of cell suspension was found to be negligible [0–10 ng/flask compared with several thousand nanograms per flask after incubation of substrate with homogenates of the renal cell suspensions], and angiotensin activity in the homogenized cell suspension prior to addition of substrate was also negligible.

Results. Effect of collagenase on renin. In view of the possibility that traces of collagenase might have remained in the renal cell suspension even after careful washing, an experiment was performed to determine whether it would affect renin activity. Collagenase in a final concentration of 0.1% was added to plasma samples of known renin content, and the samples were then incubated for 1 hr at 37°. The renin activity of plasma incubated with collagenase was found to be the same as that of plasma incubated without collagenase.

Effect of epinephrine on net renin production. Eight experiments were performed in which epinephrine was added to cell suspension samples. In all eight experiments, the suspensions that were incubated with epinephrine had higher concentrations of renin activity than did the control suspensions incubated without epinephrine (Fig. 1). "Net renin production," calculated by subtracting the nonincubated control values from the cor-

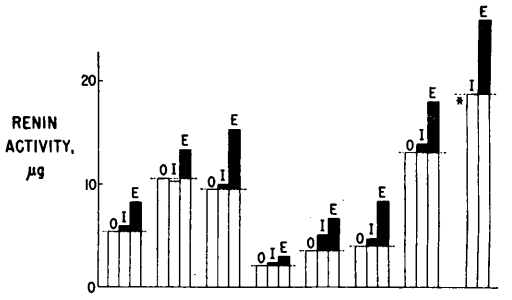

FIG. 1. Stimulation of renin production by epinephrine in renal cell suspensions: O = nonincubated controls (the quantity of renin in the cell suspensions prior to incubation). This value should be subtracted from the others to obtain "net renin production" during incubation of the cell suspensions. I = incubated control suspension; E = suspensions incubated with epinephrine (0.5 μg/ml of suspension, added at the beginning and every 5 min of the first 0.5 hr of incubation). Time of incubation was 1 hr; * = nonincubated control not available due to technical error.

responding incubated sample values, was severalfold higher in the suspensions incubated with epinephrine than in those incubated without. This rise of renin activity could not be attributed to a pressor effect of epinephrine that might have been carried over into the assay samples, since suspension samples incubated with epinephrine had no pressor activity prior to the 3-hr incubation with renin substrate. In other words, the pressor material was not present at the end of the incubation of the renal cell suspension; it was generated during the subsequent 3-hr period during which the homogenized renal cell suspension was incubated with plasma containing renin substrate. When all experiments were considered as a group, the net production of renin in suspensions incubated with epinephrine was statistically significant (Fig. 2).

Effect of norepinephrine on net renin production. Four experiments in which norepinephrine was added to the incubated cell suspension samples were performed in the same manner as those with epinephrine and yielded similar results (Fig. 3). In all four experiments net renin production was several times greater in the suspensions incubated with norepinephrine than in those incubated without this agent. As in the case of the epinephrine experiments, the cell suspensions were tested for pressor activity prior to incubation with renin substrate and were found to be inactive. The increased renin activity, therefore, was not attributable to the pressor action of norepinephrine itself. When all experiments with norepinephrine were combined, the net production of renin was statistically significantly higher in the presence than in the absence of norepinephrine (Fig. 2).

Effect of cyclic AMP on net renin production. In seven experiments, cyclic AMP was added to the cell suspension samples (Fig. 4). In all seven experiments the net production of renin was several times greater in the suspensions incubated in the presence of cyclic AMP than in the control suspensions incubated without cyclic AMP. When all the experiments with cyclic AMP were considered as a group, the net renin production was statistically higher in the presence than in the absence of cyclic AMP (Fig. 2).

Net renin production in response to various doses of stimulants. The effects of different concentrations of epinephrine, norepineph-

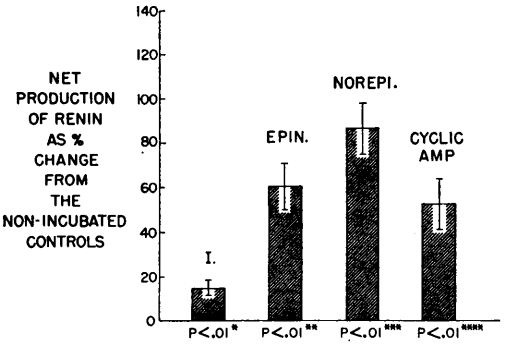

FIG. 2. Combined data as the mean percentage change in renin from the nonincubated controls. I= incubated controls; Epin. = suspensions incubated with epinephrine; Norepi. = suspensions incubated with norepinephrine; Cyclic AMP = suspensions incubated with cyclic AMP; the brackets indicate the standard errors of the means. Net renin production in the incubated control samples was statistically significant ($p<0.01$). The increases of net renin production with epinephrine, norepinephrine, and cyclic AMP over that of the incubated controls were also statistically significant ($p<0.01$). (*), I vs nonincubated control; (**), Epin. vs I; (***), Norepi. vs I; and (****), Cyclic AMP vs I.

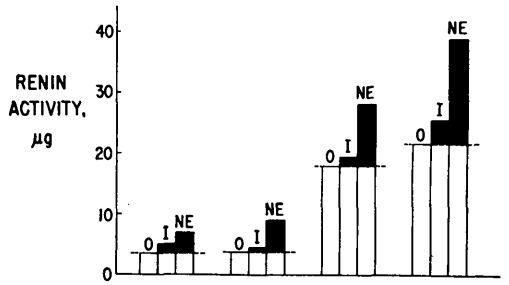

FIG. 3. Stimulation of renin production by norepinephrine in renal cell suspensions: O = nonincubated controls (the quantity of renin in the cell suspensions prior to incubation). This value should be subtracted from the others to obtain "net renin production" during incubation of the cell suspensions. I = incubated control suspensions; NE = suspensions incubated with norepinephrine (1 μg/ml of cell suspension, added at the beginning and every 5 min of the first 0.5 hr of incubation); time of incubation was 1 hr.

rine, and cyclic AMP on net renin production are shown in Fig. 5. There appeared to be a dose–response relationship with each agent.

Effect of cycloheximide on net renin production. Since cycloheximide is known to inhibit protein synthesis in mammalian cells (11), its effect on net renin production by the renal cell suspension was studied (Fig. 6). In the presence of cycloheximide, the stimulatory effect of norepinephrine on renin production was abolished.

Distribution of renin between cells and supernatant. In some experiments, after incubation with epinephrine or cyclic AMP had been completed, the supernatant was removed, and the cells were then resuspended in medium and homogenized. Renin activity was measured separately in cells and supernatant. The results of a representative experiment are shown in Table I. The renin-

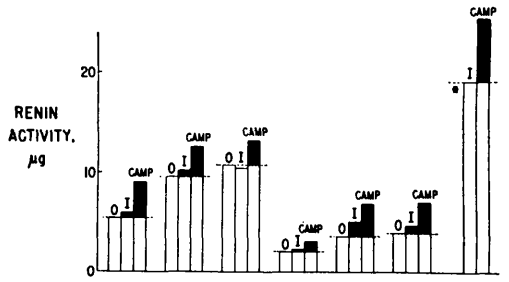

FIG. 4. Stimulation of renin production by cyclic AMP in renal cell suspensions: O = nonincubated controls (the quantity of renin in the cell suspensions prior to incubation). This value should be subtracted from the others to obtain "net renin production" during incubation of the cell suspensions. I = incubated control suspensions; CAMP = suspensions incubated with cyclic AMP (2–4 mg/ml of cell suspension, added at the beginning and at the middle of the incubation period); time of incubation was 1 hr; (*), nonincubated control not available due to technical error.

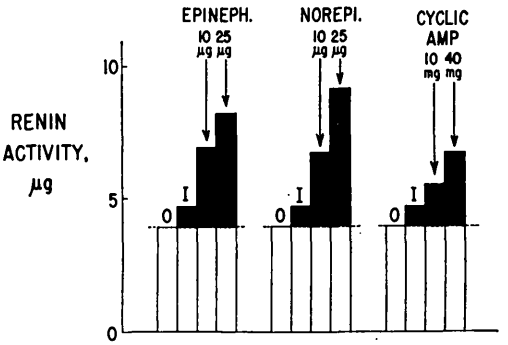

FIG. 5. Net renin production in response to various stimulants: O = nonincubated control (the quantity of renin in the cell suspension prior to incubation). This value should be subtracted from the others to obtain "net renin production" during incubation of the cell suspensions. I = incubated control suspensions; Epineph. = epinephrine; Norepi. = norepinephrine; numbers above the columns indicate quantities of each agent added per flask; time of incubation was 1 hr.

stimulating effects of epinephrine and cyclic AMP were reflected both in the cellular and supernatant fractions.

Discussion. The studies of Gordon *et al.* (3) suggested that the sympathetic nervous system has an important role in mediating the changes in net renin production that occur during circulatory adaptation to upright posture and sodium deprivation. Furthermore, the infusion of epinephrine and norepinephrine, either systemically or into the renal artery, stimulated renin secretion (3–5). It was not clear from previous studies whether sympathetic amines act directly on renin-producing cells or whether they act to cause renal afferent arteriolar constriction and thus indirectly stimulate renin secretion.

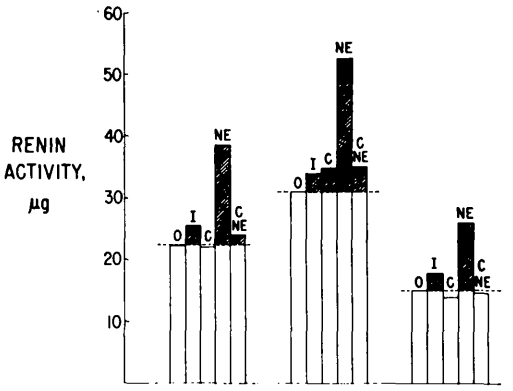

FIG. 6. Effects of norepinephrine and cycloheximide on renin production in renal cell suspensions: O = nonincubated controls (the quantity of renin in the cell suspension prior to incubation). This value should be subtracted from the others to obtain "net renin production" during incubation of the cell suspensions. I = incubated control suspensions; C = suspensions incubated with cycloheximide (0.2 mg/ml of suspension added at the beginning of incubation); NE = suspensions incubated with norepinephrine (1 μg/ml of suspension added at the beginning and every 5 min of the first 0.5 hr of incubation); CNE = suspensions incubated with both cycloheximide and norepinephrine as above; time of incubation was 1 hr.

Without excluding the latter possibility, the present study affirms the former. While relatively high concentrations of epinephrine and norepinephrine were added to the renal cell suspension, it is conceivable that these might have been within the physiologic range if one considers it possible that sympathetic nerves might secrete catecholamines in the immediate vicinity of the renin-producing cells. Support for this possibility can be derived from the recent morphologic studies of Wagermark and associates (12) who demonstrated that certain renal sympathetic nerve fibers terminate in direct contact with the granulated juxtaglomerular cells.

With the demonstration that epinephrine and norepinephrine had a direct stimulatory effect on net renin production, it became pertinent to inquire whether cyclic AMP might have a similar effect, since this agent has been considered the intracellular mediator of the action of epinephrine and norepinephrine in several other tissues (13). The observation that cyclic AMP does indeed stimulate net renin production is consistent with the view that it might be the intracellular mediator of the renin-stimulating action of the catecholamines. The supposition that the "net renin production" that occurs in the renal cell suspension actually represents new protein synthesis was supported by the observation that the addition of cycloheximide, an inhibitor of protein synthesis, largely abolished the net production of renin under the conditions of these experiments.

Summary. The preparation of a dog renal cell suspension suitable for the study of renin production is described. This suspension was utilized to study the *in vitro* effects of agents that have previously been shown to affect renin production *in vivo*. Since production and destruction of renin could not be separately quantified, data were limited to

TABLE I. Renin Activity in a Cell Suspension.[a]

Experimental variables	Renin activity (ng)		
	Supernatant	Sediment	Total/flask
1. Nonincubated control			2170
2. Incubated control	900	1550	2450
3. Epinephrine (0.5 μg/ml of suspension, added at the beginning and every 5 min of the first 0.5 hr of incubation)	1100	1900	3000
4. Cyclic AMP (2 mg/ml of suspension, added at the beginning and at the middle of the incubation period)	1110	2000	3110

[a] Aliquots were incubated with epinephrine or cyclic AMP for 1 hr; each flask contained 5 ml of renal cell suspension; at the termination of the incubation the cells (sediment) and supernatant were separately assayed for renin.

"net production" of renin. Incubated controls had only slightly higher renin content than did the nonincubated controls, but the addition of epinephrine, norepinephrine or cyclic AMP caused striking increases in "net production" of renin. The effects of these agents on "net renin production" were abolished by cycloheximide, an inhibitor of protein synthesis. It is suggested that the renin-stimulating effect of catecholamines in the intact animal might be due, at least in part, to a direct chemical action on the renal cells. Cyclic AMP may play a role as an intracellular mediator of the action of catecholamines.

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Renal Stones of Calcium Phosphate: Physicochemical Basis for Their Formation (33648)

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Nephrolithiasis resulting from calcium phosphate stones is a frequent complication of idiopathic hypercalciuria and of hypercalciuria resulting from sarcoidosis, hyperparathyroidism or prolonged treatment with Vitamin D (1, 2). New stones are usually not formed after hypercalciuria has been corrected. It has therefore been suggested that the urine of such patients is supersaturated with respect to Ca^{2+} and phosphate ions, and that this leads to precipitation and formation of calcium phosphate stones (1, 3, 4). In this communication, we present experimental verification for this hypothesis.

The calcium phosphate first formed from urine, and the phase which is stable at the normal acid pH of urine, is presumably brushite ($CaHPO_4 \cdot 2H_2O$) (5,6); at basic pH, brushite undergoes hydrolysis to octacalcium phosphate and hydroxyapatite. Thus, brushite is often found in the stones of patients with hypercalciuria. We therefore determined the state of saturation of urine with respect to brushite. This was achieved by calculating the activity product of Ca^{2+} and HPO_4^{2-} of the urine before and after incubation with brushite.

Material and Methods. The calculation of activity product of Ca^{2+} and HPO_4^{2-} requires an estimation of (i) the ionic strength (μ) of urine, (ii) the fraction of total orthophosphate represented by HPO_4^{2-}, and (iii) the activity coefficient of Ca^{2+} and HPO_4^{2-} at varying ionic strengths.

The ionic strength of urine was calculated from the formula: $\mu = 1.056\ (C_{Na^+} + C_{K^+} + C_{NH_4^+} + 4C_{Mg^{2+}} + 4C_{Ca^{2+}})$, where C is concentration in moles/liter and 1.056 is the