Role of Macula Densa in Diuretics-Induced Renin Release

Manuel Martínez-Maldonado, Rosario Gely, Edilia Tapia, and Julio E. Benabe

Diuretic therapy may enhance renin release by various mechanisms, principally contraction of extracellular fluid volume and its effects, including a fall in arterial pressure. Awake hydropenic or volume-expanded rats received diuretics (amiloride and hydrochlorothiazide) that are known inhibitors of NaCl transport beyond the macula densa; also the well-known Na⁺-K⁺-2 Cl⁻ transport system inhibitor furosemide was administered. We also evaluated the effect of a dose of ethacrynic acid (a drug that shares the same mechanism of action as furosemide but is not diuretic in the rat). The direct action of the diuretics on renin-producing cells was examined in isolated glomeruli; a rise in renin release was observed with the calmodulin inhibitor trifluoperazine (10⁻⁹ M). Renin release in intact hydropenic rats was not altered by diuretic therapy, but furosemide increased plasma renin activity in hydropenic as well as in volume-expanded rats. This demonstrates the importance of furosemide inhibition of transport in the macula densa for its renin secretory action. None of the diuretics (amiloride, hydrochlorothiazide, ethacrynic acid, or furosemide) elicited changes in renin release from glomeruli (10⁻⁶ to 10⁻³ M); amiloride and hydrochlorothiazide (10⁻⁴ to 10⁻³ M) did not change renin release from slices, but 10⁻³ M ethacrynic acid and furosemide increased renin secretion in this preparation. This suggests that an effect on the macula densa is essential in loop diuretic-mediated renin release. Because ethacrynic acid is not diuretic (at the doses used in this study) but increases renin release from slices, the study raises the possibility that a direct effect of some diuretics on tubular structures (i.e., macula densa) may cause the production of substances that are ultimately responsible for mediating renin release from renin-containing cells. (Hypertension 1990;16:261-268)

A decrease in extracellular fluid volume will increase renin release by activation of renal baroreceptors and the sympathetic systems.1,2 Other mechanisms mediate diuretic-induced renin release. The role of inhibition of NaCl transport in the macula densa of the thick ascending limb or of a direct effect on the granular juxtaglomerular cells3 by loop and other diuretics remains to be clarified. Also, renal prostaglandin (PG) production has been postulated as a mediator for the increase in renin release caused by loop diuretics, but the exact nature of this relation remains to be established.4,5 Furosemide has been shown to either stimulate or not change renin release at concentrations of 10⁻⁵ to 10⁻³ M in renal slices and renal cortical cell suspensions.6-10 Furthermore, ethacrynic acid, which is not diuretic in the rat, in a concentration of 10⁻³ M has been shown to induce renin release in superfused rat glomeruli.11 Thus, the relation between the diuretic effect and renin release, as well as the possibility of a direct in vivo action of loop diuretics on renin-producing cells, remains unclear. In addition, it is not known if, other than their effect on extracellular fluid volume,12 thiazides and potassium-sparing diuretics have effects on renin secretion.

The purpose of this study was to clarify the mechanism of renin release of several distal-acting diuretic agents and to examine the role of the macula densa in this process. To accomplish this, we used intact awake rats. In addition, isolated glomeruli and cortical renal slices were used to eliminate the action of the renal baroreceptor and nerves and examine the direct effect of diuretic agents (furosemide, ethacrynic acid, chlorothiazide, and amiloride) on the renin-producing cells in the presence (slices) and absence (glomeruli) of tubular structures (macula densa).

Methods

Experimental Animals

Male Wistar-Furth rats (National Cancer Institute, Fort Detrick, Md.) that weighed 190±10 g were used in all studies.
In Vivo Studies

Clearance studies were performed in the awake hydropenic (drinking water removed overnight for 14 hours) rat. Rats were lightly anesthetized with ether to insert polyethylene catheters (PE-50) in the left jugular vein for intravenous infusions and in the left carotid artery for blood pressure monitoring and blood sampling. The bladder was also cannulated through a small suprapubic incision. On completion of surgery, rats were placed in restraining cages, and an intravenous infusion of isotonic saline containing inulin (6 mg/ml) and para-aminohippurate (PAH) (0.1 mg/ml) at a rate of 0.03 ml/min was begun. Rats were allowed to recover for 1.5 to 2 hours before clearance determinations were done. There were three basal periods of at least 15 minutes each; blood samples (300 μl) for determination of inulin, Na⁺, K⁺, Cl⁻, and hematocrit were taken at the midpoint of each period. At the end of each basal period, a blood sample (300 μl) for basal plasma renin activity (PRA) was taken. This was followed by bolus administration of the diuretics. The dosages were as follows: amiloride hydrochloride 1 mg/kg body wt, ethacrynic acid 2.5 and 5.0 mg/kg body wt, hydrochlorothiazide 5 mg/kg body wt, and furosemide 5 mg/kg body wt. Similar doses have been used by others. Three to five experiments (n=3) in which the same protocol was used without the administration of diuretic agents did not reveal any changes in PRA over the duration of the experiments.

After a 15-minute equilibration period, three 15-minute clearances were performed as described above. Midpoint in each period, another blood sample was obtained for determination of PRA. The red blood cells of all samples were resuspended in a volume of saline (300 μl) equal to the plasma removed and were transfused back into the rat. Urine sample aliquots of each clearance period collection were used for PGE₂ and 6-keto-PGF₁α determinations. During diuretic administration, an infusion of 0.9% saline was adjusted to equal urine flow rate and prevent extracellular volume contraction. In experiments in which furosemide was administered, an equal number of hydropenic and volume-expanded rats were studied. Volume expansion was achieved by an infusion of 0.9% saline to equal 3% body weight, administered during the equilibration period and followed by an infusion that equaled urine flow during the rest of the experiment. This degree of volume expansion is known to increase delivery to the loop of Henle and distal tubule (including the macula densa).

Glomerular filtration rate and renal plasma flow were determined from the clearance of inulin and PAH, respectively. Urine and plasma sodium and potassium were measured by flame photometry; chloride was measured in a chloridometer.

In Vitro Studies

Rat renal cortical slices were prepared using a modification of the method of Henrich and Campbell and of Churchill and Churchill and Churchill et al. Briefly, rats were killed by decapitation, both kidneys quickly removed and perfused with the buffer identified below until blanching occurred; kidneys were decapsulated, placed in Krebs-Ringer bicarbonate buffer (KRB), and equilibrated with a 95% O₂-5% CO₂ mixture at 37° C. The composition of the buffer was as follows (mM): NaCl 112, NaHCO₃ 25, KCl 5, CaCl₂ 25, NaH₂PO₄ 1, MgCl₂ 0.5, glucose (0.2 g/dl), and bovine serum albumin (0.2 mg/dl). The KRB was adjusted to pH 7.4 before each
incubation was started. Glomeruli were isolated by sieving techniques that used the procedures described in detail previously. Isolation solution was identical to that described above. Figure 1 demonstrates a typical preparation. It is clear that basically there are no tubular structures attached to the glomerulus. Specifically, there are no maculae densa. Although mesangial cells have been shown to synthesize renin, they do so at a rate that is a fraction of that of granular cells. Moreover, it is not clear that renin is secreted by mesangial cells, particularly to the extent observed under basal and stimulated conditions in glomeruli. We assume therefore that renin secretion by glomeruli originates from portions of afferent arterioles that have retracted into the glomerular tuft. Thus, renin secretion from a glomerulus most likely represents the response of the granular renin-producing cells present in the retracted afferent arteriole. This has been shown to be the case by Baumbach and Skott who found, as Hammersen and coworkers had, that cells containing granules were consistently found at the vascular pole of the glomeruli and that tubular elements were never observed, thus eliminating the possibility of the presence of the macula densa in glomeruli prepared by the methods used in our studies. The incubation and sampling procedures were as described below for slices. At the end of the incubation, glomeruli were sonicated and total protein was measured by the method of Lowry et al; the results were expressed per milligram protein.

One renal cortical slice (1 mm in thickness) was obtained from the surface of each kidney using a Stadie-Riggs tissue slicer. Each renal cortical slice was divided into smaller pieces and randomly placed in 25 ml Erlenmeyer flasks (two pieces per flask) that contained 10 ml prewarmed, pre-equilibrated KRB. The flasks were placed in a constant temperature water bath (37° C) for a period of 15 minutes after which the KRB was discarded and replaced with fresh buffer. This preincubation period was followed by incubation for another 15 minutes at the end of which a sample (100 μl) was obtained for determination of basal renin concentration. The pharmacological agents were then added in a volume of 100 μl while control samples received the same volume of the solution in which the respective diuretic was dissolved. The vehicle caused no changes in the control. After 75 minutes of incubation with the diuretic, another 100 μl sample was obtained for determination of renin concentration. In all experiments, a group of slices were incubated with 10⁻⁴ M trifluoperazine, a calmodulin inhibitor that causes dramatic renin release. An aliquot of isolated glomeruli was also incubated with 10⁻⁵ M trifluoperazine. This permitted assessment of the responsiveness of the slices or glomeruli preparation to a known stimulus.

Tissue slices were dried in a vacuum oven to constant weight (mean dry weight, 1.8±0.2 mg), and this value was used to express renin production by the slices. A total of 20 slices (10 flasks with two slices each) were incubated per experiment; each experiment was performed in triplicate.

Analytical Procedures

Renin concentration in the incubation media of renal tissue slices and glomeruli was measured by determination of the amount of angiotensin I (Ang I) that is generated during 1 hour of incubation at 37° C with inhibitors of the converting enzyme in the presence of excess rat substrate (obtained from a rat nephrectomized for 24 hours) and protease inhibitors (dimercaprol 1.32% and hydroxyquinoline sulfate 2.64%). Ang I concentration was measured by radioimmunoassay (New England Nuclear, Boston, Mass.). Iodine-125-labeled Ang I was measured in a Model 110 Picker (Picker International, Bedford, Ohio) refrigerated beta/gamma counter. The values of renin concentration in cortical slices are expressed as nanograms Ang I per milligram dry tissue, and renin secretion as nanograms Ang I per milligram dry tissue per milliliter per hour.

For determination of PRA, samples were incubated as described above but in the absence of excess substrate. Values of PRA are expressed as nanograms Ang I per milliliter per hour. PGs were measured by immunoassay using iodine-125-labeled kits (New England Nuclear).

Statistical Methods

Values are expressed as mean±SEM. Paired t and Student's t test were used when appropriate; a value of p<0.05 was accepted as significant.

Results

In Vivo Studies

Effects on mean arterial pressure and hematocrit. Except in the group of rats that were expanded with saline before diuretic administration, a decrease in hematocrit was always noted by the end of the experiment (Table 1). This may have been in part because of erythrocyte losses as a result of blood sampling for analysis and to the fact that blood losses were replaced by saline. Nevertheless, the changes, although statistically significant, were minor. There were no changes in mean blood pressure after bolus injection of diuretic agents, except in the hydroperic rats receiving furosemide, suggesting that in this group the well-known vasodilator effect of furosemide had its maximal expression.

Effects of Renal Plasma Flow and Glomerular Filtration Rate

No changes in glomerular filtration rate (Table 2) were observed in any of the groups with the administration of any of the diuretics. Moreover, a fall in renal plasma flow only occurred in hydroperic rats receiving furosemide and was probably a reflection of the modest fall in systemic blood pressure.
Effects on Sodium, Chloride, and Potassium Excretion

Table 3 summarizes the effect of the diuretics on fractional excretion of electrolytes under various conditions. All agents significantly increased fractional sodium and chloride excretion except ethacrynic acid. The lack of natriuretic and chloretural effect of ethacrynic acid in the rat is well known. Hydrochlorothiazide led to similar degrees of fractional sodium and chloride excretion. The lack of natriuretic and chloride excretion during amiloride administration. Furosemide was the only agent that consistently raised PRA (Table 4). In fact, it did so even under conditions of prior volume expansion. Although volume expansion blunted furosemide-induced renin release, as compared with hydropenic rats, the response was still highly significant. None of the other agents altered renin secretion.

Effects on Plasma Renin Activity

None of the diuretic agents (Table 5) produced significant changes in the urine excretion of PGE₂.
and 6-keto-PGF₁α. No difference in the excretion of PGs was observed in response to hydropenia as compared with volume expansion in the rats that received furosemide. We did not measure PGs during amiloride-induced diuresis.

**In Vitro Studies**

**Slices.** Figure 2 shows the release of renin by cortical slices in response to a concentration of 10⁻⁴ M diuretic. Control conditions did not differ. However, as explained, renin release was significantly different at 90 minutes compared with 15 minutes in both control and diuretics at 10⁻⁴ M. By contrast, trifluoperazine at a concentration of 10⁻⁵ M caused an increase in renin release that was from 10-fold to 20-fold of that observed with any of the diuretics or control at the end of the incubation period. No response was obtained at 10⁻⁶ or 10⁻⁵ M with any of the diuretics (not shown). Higher doses (10⁻³ M) of trifluoperazine caused a dramatic rise in renin release. In contrast to the results with slices, neither furosemide nor ethacrynic acid could be shown to increase renin release in isolated glomeruli. All experiments exhibited a significant rise in renin release over 90 minutes. Nevertheless, concentrations of diuretics as high as 10⁻³ M could not be shown to differ from time controls incubated with the vehicle for the drug.

**TABLE 4. Effect of Diuretic on Plasma Renin Activity in Awake Hydropenic Rats**

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Before</th>
<th>After</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furo</td>
<td>5</td>
<td>11.9±1.0</td>
<td>45.9±5.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Furo+VE (3%)</td>
<td>5</td>
<td>3.1±0.5</td>
<td>9.8±0.5</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>EA (2.5)</td>
<td>5</td>
<td>7.2±2.6</td>
<td>15.0±6.6</td>
<td>NS</td>
</tr>
<tr>
<td>EA (5.0)</td>
<td>5</td>
<td>12.2±3.9</td>
<td>10.3±2.8</td>
<td>NS</td>
</tr>
<tr>
<td>HCTZ</td>
<td>6</td>
<td>10.4±2.8</td>
<td>17.4±6.0</td>
<td>NS</td>
</tr>
<tr>
<td>Am</td>
<td>5</td>
<td>10.3±2.4</td>
<td>12.3±1.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SEM of three basal and three experimental periods. n, number of animals; PRA, plasma renin activity; Furo, furosemide, 5 mg/kg; EA, ethacrynic acid, 2.5 and 5 mg/kg; HCTZ, hydrochlorothiazide, 5 mg/kg.

**FIGURE 2.** Plot showing renin secretion by renal cortical slices incubated in Krebs-Ringer bicarbonate buffer (KRB) equilibrated with 95% O₂-5% CO₂ mixture adjusted to pH 7.4 (see Methods for KRB composition). Time control increased significantly (p<0.05) after 90 minutes of incubation. So did all the samples incubated with diuretics. Nevertheless, none of the values when diuretics were incubated with glomeruli were different from control at either 15 or 90 minutes. (See text for further details.) AI, angiotensin I; HCZ, hydrochlorothiazide; AM, amiloride; FUR, furosemide; EA, ethacrynic acid; TFP, trifluoperazine.

Trifluoperazine 10⁻⁵ M caused a 10-fold rise in renin secretion (not shown).

**Discussion**

Renin release after the administration of diuretics may result from a reduction in extracellular fluid volume or a fall in blood pressure, 1,2 stimuli that increase renin release by affecting the intrarenal baroreceptors or renal nerve signal traffic. 30 These mechanisms most likely mediate furosemide-induced renin release in awake hydropenic rats. When given to hydropenic rats, furosemide caused a modest but significant fall in mean arterial pressure and renal blood flow, whereas glomerular filtration rate remained constant. Thus, because delivery of NaCl to the macula densa must have varied little if at all, it is unlikely that it played a role in renin secretion stimulation. In contrast, when furosemide was given to awake, volume-expanded animals, despite a small

**TABLE 5. Effects of Diuretics on Urine Excretion of Prostaglandin E₂ and 6-Ketoprostaglandin₆ in Awake Rats**

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Basal</th>
<th>Diuretic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furo</td>
<td>5</td>
<td>57±21</td>
<td>49±18</td>
<td>NS</td>
</tr>
<tr>
<td>Furo+VE (3%)</td>
<td>5</td>
<td>46±26</td>
<td>26±9</td>
<td>NS</td>
</tr>
<tr>
<td>EA (2.5)</td>
<td>5</td>
<td>51±14</td>
<td>57±11</td>
<td>NS</td>
</tr>
<tr>
<td>EA (5.0)</td>
<td>5</td>
<td>36±14</td>
<td>55±23</td>
<td>NS</td>
</tr>
<tr>
<td>HCTZ</td>
<td>6</td>
<td>67±15</td>
<td>55±18</td>
<td>NS</td>
</tr>
</tbody>
</table>

 Values are mean±SEM of three basal and three experimental periods. n, number of animals; PG, prostaglandin; Furo, furosemide; VE (3%), volume expansion, 3% body weight; EA, ethacrynic acid, 2.5 and 5 mg/kg; HCTZ, hydrochlorothiazide, 5 mg/kg.
fall in hematocrit and no change in mean arterial blood pressure and glomerular filtration rate, plasma renin activity rose sharply. One must conclude, therefore, that volume contraction did not occur; thus stimulation of the baroreceptor as a result of changes in volume or reduced arterial pressure were absent. Because glomerular filtration rate did not change and volume expansion was present, distal delivery of NaCl was, if anything, higher than normal. Clearly furosemide-induced renin release in this setting must have been the result of a direct effect on macula densa transport or on the granular renin-producing cells.

We have suggested that inhibition of NaCl transport in the region of the macula densa during furosemide diuresis is most likely responsible for initiating the signal that ultimately leads to enhanced renin secretion. The results of the experiments performed during volume expansion suggest, as we have indicated previously from studies of similar design, that furosemide causes the macula densa to perceive diminished transcellular transport of NaCl and this, in some as yet undefined way, results in renin release.

Recent studies by Briggs et al in isolated, perfused ascending limbs with a macula densa attached to its glomerulus give direct evidence for the suggestion that diminished sodium chloride transport across the macula densa induces renin secretion. Furthermore, a study by Itoh and Carretero demonstrated that furosemide added to microdissected rabbit afferent arterioles alone did not elicit any change in renin release. However, when furosemide was added to arterioles with macula densa attached, renin release increased markedly. Our experiments in vitro lend further credence to this interpretation. Previous studies from this laboratory on the effect of furosemide and ethacrynic acid in isolated dog glomeruli and the present studies with rat cortical slices and glomeruli demonstrate that none of the four diuretics used (amiloride, hydrochlorothiazide, ethacrynic acid, or furosemide) in doses of $10^{-6}$ to $10^{-4}$ M were capable of inducing renin secretion. Moreover, enhanced renin release was not observed when $10^{-3}$ M ethacrynic acid or furosemide were added to isolated glomeruli.
Neither hydrochlorothiazide nor amiloride changed renin release in intact animals or in isolated glomeruli or slices. Because their tubular site of action is beyond the macula densa and does not involve a Na⁺-K⁺-2Cl⁻ cotransporter mechanism as part of the sensing device that is ultimately responsible for the regulation of renin release. 

It is interesting that furosemide, which clearly inhibited ion transport in the thick ascending limb, was predominantly chlorothiazide. It has been proposed by some authors that reduced reabsorption of Cl⁻ is the fundamental signal (over that of Na⁺) to initiate macula densa-mediated renin release. Although such a conclusion cannot be categorically arrived at from our results, the predominantly chlorothiazide effect provides circumstantial evidence that transport of Cl⁻, such as that mediated by a Na⁺-K⁺-2Cl⁻ cotransporter, may mediate the macula densa signal for renin release.

We could not marshal sufficient evidence to indicate that renin release by furosemide in vivo or in vitro was mediated by PG secretion. This is consonant with other data obtained in this laboratory to the effect that the loop diuretic oxonolone, a Na⁺-K⁺-2Cl⁻ cotransporter inhibitor, does not alter PG production by isolated dog glomeruli nor does it cause renin secretion in that preparation.

Our studies also demonstrate that diuretics that may increase renin release in vivo are not effective in altering renin secretion if rats are maintained euvoletic, provided their major site of action is beyond the macula densa (hydrochlorothiazide and amiloride). Furthermore, amiloride, which increases renin release in hypertensive humans and in patients with congestive heart failure does not alter PG production by isolated dog glomeruli and nor does it cause renin secretion in that preparation.34

Furosemide at high doses failed to increase renin release by glomeruli, yet the increased renin secretion from slices and in awake, volume-expanded rats suggest a critical role for the macula densa and the Na⁺-K⁺-2Cl⁻ cotransporter as part of the sensing device that is ultimately responsible for the regulation of renin release.

References


KEY WORDS • chemoreceptors • diuretics • renin
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