Role of the Macula Densa in Renin Release

SADAYOSHI ITOH AND OSCAR A. CARRETERO

SUMMARY To examine the role of the macula densa in renin release, afferent arterioles alone or afferent arterioles with the macula densa attached were microdissected from rabbit kidney and incubated in Medium 199 for two consecutive 30-minute periods. Renin concentration in the medium was measured using partially purified rabbit angiotensinogen. Renin release rate over 1 hour from a single arteriole (or an arteriole with macula densa) was calculated and expressed as nanograms of angiotensin I generated per hour per arteriole (or arteriole with macula densa) per hour incubation (ng of ANG I • hr⁻¹ • Af⁻¹ • hour). Basal renin release rate from afferent arterioles was 0.69 ± 0.13 ng of ANG I • hr⁻¹ • Af⁻¹ • hour (mean ± SEM, n = 9) and remained stable for 60 minutes. Basal renin release rate from arterioles with macula densa was 0.25 ± 0.03 ng of ANG I • hr⁻¹ • Af + MD⁻¹ • hour (n = 9), which was significantly lower (p < 0.025) than that from afferent arterioles alone. When furosemide (1.5 × 10⁻³ M) was added to afferent arterioles alone, no significant change in renin release was observed (percent change from control; 24.8 ± 29.9%; p > 0.05, n = 6). When furosemide was added to arterioles with macula densa attached, however, renin release increased by 387 ± 46% (n = 7; p < 0.001). After pretreatment with indomethacin, a cyclooxygenase inhibitor, furosemide still increased renin release from 0.17 ± 0.03 to 0.60 ± 0.10 ng of ANG I • hr⁻¹ • Af + MD⁻¹ • hour (n = 4; p < 0.05); however, indomethacin pretreatment reduced both the basal renin release rate and the absolute change in renin release induced by furosemide. We conclude that (1) the macula densa inhibits renin release in this preparation, (2) the macula densa plays a central role in furosemide-induced renin release, and (3) while the prostaglandin system is not essential for furosemide-induced renin release, it may be a modulating factor. (Hypertension 7 [Suppl 1]: 1-49-1-54, 1985)

KEY WORDS • rabbit • microdissection • afferent arteriole • furosemide • prostaglandin • indomethacin • sodium concentration

THE macula densa, a specialized portion of the distal tubule, is in close contact with the vascular component of the juxtaglomerular apparatus. Because of this intimate anatomical relationship, it has been postulated that renin release may in some way be controlled by the ionic composition of the tubular urine at the macula densa. There is, however, no direct evidence demonstrating a role for the macula densa in the regulation of renin release.

In an attempt to relate the rate of renin release to changes in sodium metabolism, many physiological and pharmacological perturbations have been used to alter renal sodium excretion. Among the pharmacological tools used have been the so-called loop diuretics, which are known to inhibit active sodium chloride transport in the thick ascending limb of the loop of Henle. These diuretics increase renin release. Although the macula densa mechanism has been speculated to be involved in these diuretic-induced enhancements of renin release, no conclusive study has yet been reported.

To examine the role of the macula densa in renin release, we adapted a microdissection technique to isolate rabbit afferent arterioles alone or afferent arterioles with macula densa attached. Using these two in vitro preparations, we studied whether the presence of the attached macula densa affects renin release, whether the macula densa plays a role in furosemide-induced renin release, whether the prostaglandin system is involved in furosemide-induced renin release mediated by the macula densa, and, finally, whether the reduction of sodium chloride concentration in the incubation medium alters renin release.

Methods

Isolation and Incubation of Afferent Arterioles and Afferent Arterioles with Macula Densa

Young male New Zealand white rabbits (1.5-2.5 kg) maintained on standard rabbit chow (Ralston Purina Co, St. Louis, MO) and tap water ad libitum were anesthetized with pentobarbital (40 mg/kg i.v.; Abbott...
Laboratories, North Chicago, IL) and given an intravenous injection of heparin (500 U, Elkins-Sinn, Inc, Cherry Hill, NJ). The left kidney was perfused in situ with cold oxygenated (95% O2, 5% O2) Medium 199 (Gibco Laboratories, Grand Island, NY), containing 0.1% bovine serum albumin (BSA; Schwartz/Mann, Orangeburg, NY). Then the kidney was removed and sliced along the corticomedullary axis. Slices were placed in ice-cold Medium 199 and microdissected at 4°C under a stereomicroscope at the magnification up to ×100.

Figure 1 shows a photograph of a microdissected arteriole and an arteriole with macula densa attached along with a schema of the structures in the photograph. The afferent arterioles were severed from interlobular arteries and glomeruli with hypodermic needles (26-30 gauge). Care was taken to avoid distorting arterioles or disrupting vascular poles during the microdissection of arterioles with macula densa, the thick ascending limb of the loop of Henle and the distal convoluted tubule were cut off at a point less than 50 μm from the macula densa.

Approximately five afferent arterioles and (200-250 μm in length) afferent arterioles with macula densa each were microdissected from the outer half of the cortex in 90 minutes. The microdissected structures in each group were transferred into a small plastic ladle with a nylon mesh bottom (54 μm; Tetko, Inc., Elmstead, NY). These ladles with afferent arterioles or afferent arterioles with macula densa were preincubated in 7 ml of oxygenated Medium 199 with 0.1% BSA for 35 minutes. Following preincubation, each ladle was rinsed, blotted, and transferred into a plastic microtube containing 100 μL of Medium 199 with 0.1% BSA for 35 minutes. Following preincubation, each ladle was rinsed, blotted, and transferred into a plastic microtube containing 100 μL of Medium 199 with 0.1% BSA. The gas layer above the incubation medium was replaced with 95% O2, 5% CO2, and the microtubes were covered tightly. The microdissected structures were incubated for 30 minutes, and then the ladles were transferred to fresh media for another 30-minute incubation period. Incubation medium left in the microtube was frozen (−20°C) until the renin assay was performed. After serial incubations were completed, it was confirmed microscopically that all microdissected structures remained in the ladle. Microdissected structures were placed in 1 ml of Medium 199 with 0.1% BSA and immediately frozen. After freezing and thawing was repeated five times, the tissue samples were stored frozen until tissue renin content was determined.

Experimental Protocol

Time Control

Afferent arterioles and arterioles with macula densa were incubated in Medium 199 with 0.1% BSA for two 30-minute periods. Medium 199 had the following composition: Na+, 133 mEq/L; K+, 5.4 mEq/L; Cl−, 126 mEq/L; Ca2+, 3.6 mEq/L; HCO3−, 14.9 mM; H2PO4−, 1.0 mM.

Furosemide

Afferent arterioles and arterioles with macula densa were incubated in Medium 199 with 0.1% BSA for the first (control) period and then placed in medium containing furosemide (Elkins-Sinn, Inc.) for the second (experimental) period. Two concentrations of furosemide were tested: 1.5 × 10−4 M and 3 × 10−4 M.

Pretreatment with Prostaglandin Synthesis Inhibitor

Indomethacin (Sigma Chemical, St. Louis, MO), a cyclooxygenase inhibitor, was dissolved in saline containing 115 mg/ml of sodium carbonate at a final concentration of 3.57 mg/ml. Rabbits received an intravenous injection of indomethacin (5 mg/kg) 1 hour before the kidney was removed. In addition, indomethacin was added to all the media at a final concentration of 5 μg/ml. For the vehicle control, rabbits received an intravenous injection of vehicle (1.4 ml/kg) 1 hour before the kidney removal, and 140 μL of vehicle was added to every 100 ml of medium. Microdissection and incubation were performed as described, and furosemide (1.5 × 10−4 M) was added to incubation media during the second incubation period.

Reduced Sodium Chloride Concentration

Afferent arterioles and arterioles with macula densa were incubated in medium with low sodium chloride concentration for the experimental period. This medi-
The medium was identical to that described in Time Control except that it had a lower sodium chloride concentration (Na⁺, 17 mEq/L; Cl⁻, 10 mEq/L). Mannitol was added to the low sodium medium to keep the osmolality at 295 mosm/kg.

Analysis of Renin Activity

Renin concentration in the medium and tissue was determined as previously described. Briefly, arterioles alone or with macula densa were placed in Medium 199 plus 0.1% BSA then frozen and thawed five times and stored frozen until determination of renin content. Incubation media or tissue samples were incubated with partially purified rabbit substrate equivalent to 1,000 ng of angiotensin I (ANG I) at 37°C for 3 hours (pH 6.5). Generated ANG I was measured by radioimmunoassay. Substrate was prepared by ammonium sulfate fractionation of the plasma obtained from rabbits nephrectomized 48 hours previously. Substrate had specific activity ranging from 300 to 700 ng of ANG I/mg of protein, and had no detectable renin or angiotensinase activity.

Renin release rate was calculated as nanograms of ANG I generated per hour per arteriole (or arteriole with macula densa) per hour incubation of arteriole (or arteriole with macula densa) and expressed as ng of ANG I·hr⁻¹·Af⁻¹ (or Af+MD⁻¹)/hour. Tissue renin content of a single arteriole (or an arteriole with macula densa) was calculated and expressed as ng of ANG I·hr⁻¹·Af (or Af+MD).

All data were expressed as mean ± SEM. Student's paired and unpaired t test were used for the statistical evaluation; p < 0.05 was considered to be significant.

Results

Time Control

Renin release rates from afferent arterioles and afferent arterioles with macula densa were initially 0.69 ± 0.13 ng of ANG I·hr⁻¹·Af⁻¹/hour and 0.25 ± 0.03 ng of ANG I·hr⁻¹·Af+MD⁻¹/hour respectively, and they remained stable during the next incubation period (Table 1). Although tissue renin content of afferent arterioles with macula densa was greater (p < 0.025) than that of afferent arterioles alone, renin release rate was significantly less (p < 0.025) in afferent arterioles with macula densa (Figure 2; see Table 1). Thus, the ratio of renin release to tissue renin content was significantly smaller (p < 0.0005) in afferent arterioles with macula densa (0.35 ± 0.05%) than in afferent arterioles alone (2.5 ± 0.46%). There was, however, a significant correlation between tissue renin content and renin release rate in both groups (see Figure 2).

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**Table 1** Effect of Furosemide on Renin Release from Afferent Arterioles with and without Macula Densa

<table>
<thead>
<tr>
<th>Group</th>
<th>Renin release rate (ng of ANG I·hr⁻¹·Af⁻¹ [or Af+MD⁻¹]/hr)</th>
<th>Tissue renin content (ng of ANG I·hr⁻¹·Af [or Af+MD])</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control period</td>
<td>Experimental period</td>
</tr>
<tr>
<td>I</td>
<td>Afferent arterioles</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Furosemide</td>
<td>15 × 10⁻³ M</td>
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<td></td>
<td>30 × 10⁻⁴ M</td>
<td>5</td>
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<tr>
<td>II</td>
<td>Afferent arterioles with macula densa</td>
<td></td>
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<tr>
<td>1</td>
<td>Control</td>
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<td>Furosemide</td>
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<td>30 × 10⁻⁴ M</td>
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</table>

Values are mean ± SEM

\( n = \text{number of experiments, ANG I = angiotensin I, Af = afferent arteriole; MD = macula densa} \)

\( * = p < 0.025, † = p < 0.001 \) compared with the control period; ‡ = p < 0.05; § = p < 0.025 compared with afferent arteriole alone (control versus control and experiments versus experiments).
Furosemide

When afferent arterioles alone were exposed to furosemide at $1.5 \times 10^{-3}$ M, no significant change in renin release was observed (percent change from control; $24.8 \pm 29.9\%$; $p > 0.05$). When afferent arterioles with macula densa were exposed to the same concentration of furosemide, however, renin release rate increased significantly ($p < 0.001$) by $347\%$ (see Table 1; Figure 3). A lower concentration ($3.0 \times 10^{-4}$ M) of furosemide also increased renin release only from afferent arterioles with macula densa (Table 1).

Pretreatment with Prostaglandin Synthesis Inhibitor

Because vehicle treatment did not affect the renin response to furosemide ($0.31 \pm 0.04$ and $1.35 \pm 0.3$ ng of ANG I $\cdot$ hr$^{-1} \cdot$ Af $+\$ MD$^{-1}$/hour in control and experimental period respectively; $n = 4$), data from the vehicle-treated and nontreated groups were pooled and served as control. When afferent arterioles with macula densa were pretreated with indomethacin, furosemide at $1.5 \times 10^{-3}$ M still increased renin release significantly ($p < 0.05$) from $0.17 \pm 0.03$ to $0.60 \pm 0.10$ ng of ANG I $\cdot$ hr$^{-1} \cdot$ Af $+\$ MD$^{-1}$/hour (Figure 4). Indomethacin pretreatment reduced basal renin release from $0.32 \pm 0.03$ in the control group to $0.17 \pm 0.04$ ng of ANG I $\cdot$ hr$^{-1} \cdot$ Af $+\$ MD$^{-1}$/hour in the indomethacin pretreated group ($p < 0.01$). The absolute increase ($\Delta$) in renin release after furosemide was $1.23 \pm 0.13$ in the control group and $0.43 \pm 0.11$ ng of ANG I $\cdot$ hr$^{-1} \cdot$ Af $+\$ MD$^{-1}$/hour in the indomethacin pretreated group ($p < 0.01$).

Reduced Sodium Chloride Concentration

When the sodium chloride concentration of the medium was reduced, renin release rate from afferent arterioles decreased from $0.61 \pm 0.13$ to $0.29 \pm 0.11$ ng of ANG I $\cdot$ hr$^{-1} \cdot$ Af $+\$ MD$^{-1}$/hour ($p < 0.05$). Renin release rate from afferent arterioles with macula densa was not affected by the reduction of sodium chloride concentration ($0.25 \pm 0.09$ and $0.22 \pm 0.04$ ng of ANG I $\cdot$ hr$^{-1} \cdot$ Af $+\$ MD$^{-1}$/hour in control and experimental periods respectively; Figure 5).

Discussion

Recently, we have shown that the microdissected afferent arteriole is a viable and suitable preparation for the study of renin release. In the present study, we further extended this preparation to a model in which renin release can be studied in the presence and absence of macula densa. The results suggest that (1) the macula densa has an inhibitory action on renin release, (2) the macula densa may mediate furosemide-induced enhancement of renin release, and (3) the prostaglandin system may not be an essential role in the modulation of renin release.
factor for the furosemide-induced renin release mediated by the macula densa but may modulate the absolute change in renin release.

Renin release in vitro is reported to be 2 to 10% of tissue renin content each hour. The renin release rate from afferent arterioles alone (2.5%) in the present study is in good agreement with these previous reports; however, renin release rate from afferent arterioles with macula densa attached (0.35%) was significantly lower than that from the afferent arterioles alone. As this preparation of afferent arterioles with macula densa has only very short segments of the thick ascending limb of the loop of Henle and distal convoluted tubule, tubular reabsorption would have a negligible influence on the sodium chloride concentration at the luminal side of macula densa. Thus, the sodium chloride concentration at the luminal side of macula densa would be equal to that of the incubation medium (Na+, 133 mEq/L; Cl-, 126 mEq/L) and higher than in vivo early distal tubular urine (~60 mEq/L). According to the hypothesis of Vander, renin release rate is inversely related to the sodium load to the macula densa and the link between sodium load and renin release is the rate of sodium transport by macula densa. It would be compatible with this hypothesis to speculate that the inhibition of renin release by the macula densa observed in the present study may be due to the high sodium chloride concentration of the incubation medium (and high sodium chloride transport by macula densa).

To test whether the inhibition of renin release by the macula densa may be due to a high sodium chloride concentration at the macula densa, we reduced the sodium chloride concentration of the incubation medium while the osmolality was kept constant at 295 mosm/kg. The reduction of sodium chloride concentration decreased renin release from afferent arterioles, whereas it did not affect renin release from afferent arterioles with macula densa. This difference in the renin secretory response may be related to an interaction between the macula densa and juxtaglomerular cells. It may be speculated that a macula densa mechanism was activated by the low sodium chloride concentration, which in turn antagonized the inhibitory effect of low sodium chloride concentration on renin release from juxtaglomerular cells. It could also be possible that a low sodium chloride concentration blunted the responsiveness (renin release) of juxtaglomerular cells to a signal generated by the macula densa. In either case, the effect of low sodium chloride concentration on a macula densa mechanism may already have been achieved by a low sodium chloride concentration at the macula densa. Further stimulation by furosemide would then cause only a small increase in renin release. On the other hand, in our preparation, renin release may already have been suppressed by a high sodium chloride concentration at the macula densa before the administration of furosemide. Subsequent exposure to furosemide would then have resulted in an efficient activation of the macula densa mechanism.

The mechanisms by which furosemide stimulates renin release have been studied extensively. Vander and Carlson reported that in anesthetized dogs, a small but definitely natriuretic dose of furosemide (0.1 mg/kg) stimulated renin release only when salt depletion was allowed to occur and that the enhanced renin release induced by larger doses (0.5 and 2.5 mg/kg) was not affected by the replacement of salt and water losses. Based on these observations, they hypothesized that higher doses of furosemide stimulated renin release by directly inhibiting sodium transport at the macula densa. In this respect, it is of interest to note that the cells of the macula densa are morphologically similar to the epithelial cells of the thick ascending limb of the loop of Henle, where furosemide is known to inhibit active sodium chloride transport. Although there have been a number of reports in favor of Vander's hypothesis, no previous study has reported an increase in renin release induced by furosemide. In the present study, furosemide increased renin release only in the presence of the macula densa. This observation may be considered among the first direct evidence indicating that the macula densa mediates furosemide-induced renin release. The mechanism by which furosemide increases renin release could be due to the blockade of sodium chloride transport at the macula densa, as hypothesized by Vander and Carlson.

Over the past decade there have been a number of reports that support and refute a role of the prostaglandin system in the action of furosemide on renin release. In some in vivo studies, renin release induced by furosemide was attenuated by pretreatment with cyclooxygenase inhibitors, whereas others have reported no effect of this pretreatment on furosemide-induced renin release. Several factors may account for the discrepancies in these results, for example, the presence or absence of anesthesia, hemodynamic changes induced by furosemide, and changes in salt and water balance induced by cyclooxygenase inhibitors. Because our preparation is independent of such influences, the results could be interpreted directly. Although the inhibition of prostaglandin synthesis by indomethacin reduced basal renin release rate and the absolute increase in renin release, furosemide still enhanced renin release significantly. This result suggests that the prostaglandin system is not an essential factor for the furosemide-induced renin release but may modulate the response. As it has been shown immunohistochemically that cyclooxygenase is present in the afferent arteriole but not
in the macula densa, the modulation of the renin response to furosemide may be a function of prostaglandin synthesis in afferent arteriole. It could be argued that the failure of indomethacin pretreatment to prevent the furosemide-induced renin release was due to an incomplete blockade of prostaglandin synthesis, although similar treatment (5 mg/kg i.v. 40 minutes before the removal of kidney and 1 µg/ml in incubation medium) to that presently used has been reported to efficiently block prostaglandin synthesis in rabbit kidney cortical slices. Moreover, the same pretreatment used in the present study blocked the increase in renin release induced by arachidonic acid (1.2 x 10^-6 M) in afferent arterioles alone (unpublished data, 1984). Although this evidence suggests that this pretreatment efficiently inhibited cyclooxygenase, an action of indomethacin other than cyclooxygenase inhibition cannot be ruled out.

In conclusion, our results provide direct evidence that the macula densa portion of tubule is actually involved in the regulation of renin release. The results of the present study suggest that (1) the macula densa may have an inhibitory role on renin release under conditions of high sodium chloride concentration, (2) the macula densa mediates furosemide-induced renin release, and (3) the prostaglandin system is not essential for furosemide-induced renin release mediated by the macula densa; rather it appears to be a modulating factor.

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