Nonprostanoid Endothelium-Derived Factors Inhibit Renin Release

William H. Beierwaltes and Oscar A. Carretero

Although endothelium-derived prostaglandin I₂ stimulates renin release, exogenous endothelium-derived relaxing factor (EDRF) can inhibit it. To characterize the role of EDRF as an endogenous regulator of renin release, we inhibited or stimulated its production in rat renal cortical slices in vitro. Renin concentration in the incubation medium was determined by radioimmunoassay for angiotensin I (Ang I) generation. N⁶-Monomethyl-L-arginine (LNMMA) (10⁻⁴ M), which blocks EDRF formation, significantly enhanced basal renin release from kidney slices by more than 50% in control medium (40.0±14.3 ng Ang I/hr/mg/30 min; p<0.01) or in medium treated with 1.6×10⁻⁵ M meclofenamate (50.8±8.4 ng Ang I; p<0.025). Isoproterenol (10⁻⁵ M)-stimulated renin release (40.0±14.3 ng Ang I; p<0.02) was not modified by LNMMA; addition of L-arginine (10⁻⁵ M), the precursor of EDRF, did not change basal but blocked isoproterenol stimulation of renin. Nitroprusside (10⁻⁵ M) completely reversed melittin-stimulated renin release. Endothelin-1, an endothelium-derived vasoconstrictor, inhibits renin release and stimulates EDRF and prostaglandin synthesis. To determine whether any of the renin-inhibiting effect of endothelin-1 was due to its stimulation of EDRF, we compared the effect of endothelin-1 on cortical slices with and without EDRF inhibition. Endothelin-1 (10⁻⁷ M) decreased renin by 36.7±10.9 ng Ang I (p<0.01) compared with controls, and the response was the same after either LNMMA or hemoglobin treatment. Together, these results suggest that endogenous EDRF released from the renal microvasculature inhibits both basal and stimulated renin release and that endothelin-1 inhibits renin independently of coincident EDRF. Thus, each of these nonprostanoid endothelium-derived factors may act independently as inhibitory modulators of renin release. (Hypertension 1992;19[suppl II]:II-68–II-73)
servation attributed to the associated increase in intracellular calcium as an inhibitory second messenger. On the other hand, it also has been shown that endothelin stimulates endothelial production of EDRF, which could also contribute to inhibition of renin release. The second goal of our study was to determine whether any component of the renin inhibition caused by endothelin could be attributed to the action of EDRF.

These studies, designed to clarify the role of endogenous renal EDRF in modulating renin release, have been carried out in vitro with rat renal cortical slices to eliminate the confounding effects of perfusion pressure, blood flow, and renal nerves. These studies have been designed to test the role of the primary EDRF, nitric oxide. However, because we are unable to actually measure nitric oxide in our preparations, we will refer to it with the more traditional name of EDRF.

Methods

Tissue Preparation

Renal cortical slice preparation. All procedures were carried out with in vitro incubation of rat renal cortical slices as described previously. In summary, fasting male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, Mass.) weighing 250–350 g were anesthetized with intraperitoneal sodium pentobarbital (Nembutal, Abbott, Chicago; 5 mg/100 g body wt). The kidneys were exposed by a midventral incision and flushed free of blood by retrograde perfusion with an aortic cannula. All procedures were approved by and in accordance with institutional guidelines for the care and use of laboratory animals.

All studies were performed with the bicarbonate buffer medium of Churchill and Churchill, which contains (mM) NaCl 125, NaHCO3 19, KCl 4, CaCl2 2.6, NaH2PO4 1.2, MgSO4 0.8, and glucose 36. It was equilibrated with 95% O2-5% CO2 to a pH of 7.4 at a temperature of 37°C. When the kidneys were clear of blood, they were decapsulated, excised, and split longitudinally, and two 500-μm slices were cut from the lateral outer surface of each half using a Stadie-Riggs microtome. Slices from each half were paired for concurrent control and experimental incubations; in a 25-ml Erlenmeyer flask in 10 ml medium containing 0.1 g/100 ml heat-inactivated bovine serum albumin (Difco Laboratories, Detroit, Mich.). The flasks were continuously gassed with 95% O2-5% CO2 at 37°C and shaken at approximately 1 revolution/sec in a Precision water bath (GCA, Chicago). After the samples had been equilibrated for 30 minutes, a 200-μl aliquot was taken for assay of renin concentration. The experimental agent or its vehicle was then added and a second 30-minute period run, after which a second 200-μl aliquot was taken to assay renin concentration. Samples were centrifuged and frozen for analysis. The tissue was removed, oven-dried for 24 hours at 56°C, and weighed. All results were corrected by milligrams dry weight. Renin release was determined to be the difference in concentration between collections obtained at 30 and 60 minutes incubation.

Experimental Protocols

Renal EDRF-mediated inhibition of renin release. We tested the effect of inhibiting EDRF synthesis on basal renin release in the absence and presence of cyclooxygenase inhibition. Renal cortical slices were incubated in vehicle medium or with 10-4 M N0-monomethyl-L-arginine (LNMMA) (Calbiochem Corp., La Jolla, Calif.). These protocols were repeated with the addition of 1.6×10-5 M meclofenamate (Warner-Lambert Co., Ann Arbor, Mich.) with either vehicle or LNMMA. This concentration inhibits PG synthesis in renal cortical slices in vitro. We further tested the influence of two different inhibitors of EDRF on 10-5 M isoproterenol-stimulated renin release. This protocol had three different permutations, including isoproterenol stimulation of renin 1) in control medium (vehicle), 2) after preincubation with 10-4 M LNMMA, or 3) after preincubation with 10-5 M methylene blue (Sigma Chemical Co., St. Louis, Mo.).

In a third set of experiments, we tested the effect of preincubation with L-arginine, the substrate for EDRF formation. Cortical slices were treated with 10-3 M isoproterenol either in control medium or after preincubation with 10-5 M L-arginine hydrochloride (Sigma). Additional controls were carried out with the inactive isomer of the substrate, D-arginine, at 10-2 M (Sigma).

Nonendothelium-derived cGMP-mediated inhibition of renin. To further show that cGMP-mediated vaso-dilation inhibits renin release, we incubated cortical slices with Na+-nitroprusside (Elkins-Sinn, Inc., Cherry Hill, N.J.) at concentrations of 10-7, 10-5, and 10-3 M. The effect of nitroprusside on basal renin release was compared with its effect on 10-3 M melittin-stimulated (endogenous PG-mediated) renin release. As with isoproterenol, PG-stimulated renin release is considered to be mediated by stimulating cyclic AMP (cAMP).

Endothelin and renin release. Experiments were run to determine whether the inhibitory effect of endothelin on renin release was in part due to its reported stimulation of EDRF. We added 10-7 M endothelin-1 (Peninsula Laboratories Inc., Belmont, Calif.) to renal cortical slices or to slices pretreated with one of two inhibitors of EDRF: 10-4 M LNMMA or 10-8 M hemoglobin. These latter experiments used crystallized rat hemoglobin (Sigma No. H3883). The incubation flasks were light sealed in foil and the experiments run in darkness because of the photosensitivity of the hemoglobin. The incubations using endothelin were paired with similarly treated controls without endothelin. In these studies, we measured only renin release.
These studies depend on interpretation of pharmacological interventions in vitro. Although many of these tools are well documented, their specificity can always be questioned. The strength of these studies lies in the consistent pattern of results, often using more than one experimental manipulation to achieve each conclusion, to support the hypothesis.

**Analytical Methods**

The renin concentration in the buffer medium was determined by a radioimmunoassay for generation of angiotensin I (Ang I) in the presence of excess exogenous rat angiotensinogen, based on the method of Haber et al\(^1\) as described previously.\(^2\) Renin concentration was corrected by the dry weight of tissue in milligrams. Renin release was determined by subtraction of the amount released over the initial 30-minute preincubation period from the final 30-minute incubation period and is presented in nanograms Ang I per hour per milligram per 30 minutes. All experiments were compared with concurrent vehicle controls using tissue from the opposite side of the same kidney.

Data are presented as the arithmetic mean±1 SEM. Changes in renin release over time were analyzed by Student's paired t test, comparing the tested slices with matched controls from the same kidney. Multiple comparisons in similar protocols were evaluated by analysis of variance with Bonferroni's adjustment. Comparisons were considered significantly different at a value or adjusted value of \(p<0.05\).

**Results**

**Renal EDRF-Mediated Inhibition of Renin Release**

Figure 1 shows the effect of LNMMA on basal renin release from kidney slices incubated in control medium or meclofenamate. Over the initial 30 minutes of incubation, LNMMA increased basal renin release from cortical slices by 52% \((p<0.01)\) compared with renin from slices in the control medium (108.3±11.5, \(n=16\), versus 71.2±6.2 ng Ang I/hr/mg/30 min, \(n=38\), respectively). When slices were preincubated with meclofenamate, there was a tendency for basal renin release to decrease, but this was not significant. As in previous results, LNMMA increased basal renin release in meclofenamate-treated slices by 51% \((p<0.025)\) compared with slices in meclofenamate but without LNMMA (88.6±10.7, \(n=24\), versus 58.8±3.4 ng Ang I/hr/mg/30 min, \(n=43\), respectively).

Table 1 presents the effect of preincubating cortical slices with the precursor of EDRF, L-arginine. Under control conditions, we found that isoproterenol caused a significant increase in renin release that could be completely reversed in the presence of L-arginine. Also, as seen in the table, the L-arginine–treated samples tended to exhibit higher basal renin release than untreated samples, although not significantly so. However, when we pooled a larger data sample, this trend was reversed, although still not significantly different release. The values for isoproterenol-stimulated minus vehicle control incubations show that isoproterenol caused significant stimulation of renin in each permutation. Although there clearly appears to be a trend for isoproterenol-stimulated renin to be enhanced after EDRF inhibition (by 20% or 50%, respectively), neither the stimulation in the presence of LNMMA nor that seen with methylene blue was significantly different from control values.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Isoproterenol</th>
<th>Isoproterenol minus vehicle</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ((n=10))</td>
<td>69.4±11.6</td>
<td>109.4±19.8</td>
<td>40.0±14.3</td>
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<tr>
<td>LNMMA ((n=12))</td>
<td>75.0±11.6</td>
<td>125.8±14.4</td>
<td>50.8±8.4</td>
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<tr>
<td>Methylene blue ((n=12))</td>
<td>52.7±10.4</td>
<td>112.9±17.2</td>
<td>60.2±9.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM in nanograms angiotensin I per milliliter per milligram per 30 minutes incubation. Values for isoproterenol minus vehicle represent differences from paired samples. LNMMA, \(N^0\)-monomethyl-L-arginine.
Beierwaltes and Carretero  Endothelial Inhibition of Renin  II-71

**TABLE 2. Effect of L-Arginine on Isoproterenol-Stimulated Renin Release**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Isoproterenol</th>
<th>Isoproterenol minus vehicle</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=12)</td>
<td>30.8±4.4</td>
<td>63.4±7.0</td>
<td>33.2±6.5</td>
<td>&lt;0.001</td>
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<tr>
<td>L-Arginine (n=11)</td>
<td>46.6±5.0</td>
<td>47.9±5.9</td>
<td>3.1±6.9</td>
<td>NS</td>
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<tr>
<td>D-Arginine (n=11)</td>
<td>47.3±10.1</td>
<td>67.5±8.4</td>
<td>31.3±6.9</td>
<td>&lt;0.001</td>
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</table>

Values are mean±SEM in nanograms angiotensin I per milliliter per millgram per 30 minutes incubation. Values for isoproterenol minus vehicle represent differences from paired samples. LNMMA, N°-monomethyl-L-arginine.

(45.8±4.3 ng Ang I/ml/mg/30 min, n=34, in controls versus 37.9±3.9 ng Ang I/ml/mg/30 min, n=23, with L-arginine). Additionally, when we used D-arginine in similar experiments instead of L-arginine, we found that it had no effect on either basal or isoproterenol-stimulated renin release.

**Na⁺ Nitroprusside Effect on Renin Release**

Table 3 presents the concentration-dependent effect of nitroprusside on basal and melittin-stimulated renin release. Increasing concentrations of nitroprusside tended to decrease both basal and melittin-stimulated renin release. With 10⁻⁵ M nitroprusside, the stimulatory effect of melittin was completely inhibited.

**Endothelin and Renin Release**

In incubated renal cortical slices, addition of 10⁻⁷ M endothelin resulted in 50% suppression of basal renin release (p<0.01) (Figure 2). When paired control incubations were subtracted from endothelin-treated samples, renin release had decreased by 36.7±10.9 ng Ang I/hr/mg/30 min (n=9; p<0.01). When cortical slices were pretreated with either of two inhibitors of EDRF, the degree of suppression of basal renin by 10⁻⁷ M endothelin was the same as without inhibitors. With hemoglobin pretreatment, endothelin decreased renin release by 33.7±13.2 ng Ang I/hr/mg/30 min (n=10; p<0.05) compared with hemoglobin treatment alone. When pretreated with LNMMA, endothelin decreased renin release by 23.8±4.9 ng Ang I/hr/mg/30 min (n=9; p<0.005) compared with LNMMA alone.

**Discussion**

We have found that endogenous renal EDRF can inhibit renin release. Our studies suggest that renal EDRF provides tonic inhibition of basal renin release in vitro independently of PG synthesis. In addition, when the inhibitory signal of EDRF is amplified by addition of its substrate, L-arginine, EDRF reverses isoproterenol-stimulated renin release. However, we have found no evidence that in vitro inhibition of renin release by endothelin can be attributed in any way to its stimulation of EDRF.

The endothelium is a rich source of vasoactive factors and has been suggested as an important regulator of renal vascular resistance and renal function. Inhibition of EDRF formation by the precursor antagonist LNMMA in vivo results in increased blood pressure and decreased renal blood flow. This has been attributed to suppression of the tonic vasodilator action of endogenous EDRF. Our results using LNMMA in vitro suggest that similar tonic formation of EDRF may reduce basal renin release.

Many of the stimuli that evoke EDRF production also stimulate the endothelium to synthesize PGI₂. Because this is an established stimulus for renin release that could confound our results, we repeated our studies using cyclooxygenase inhibition. However, we found no significant difference and still observed the same 50% increase as before. Romero et al²⁰ have hypothesized that EDRF could function as the intrinsic renal baroreceptor, modulating renin in response to renal perfusion pressure. Although our preparation eliminates pressure as a factor, the

<table>
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<tr>
<th></th>
<th>Vehicle</th>
<th>10⁻⁷ M</th>
<th>10⁻⁴ M</th>
<th>10⁻³ M</th>
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<td>Basal renin release</td>
<td>42.4±9.0</td>
<td>27.5±5.8</td>
<td>22.5±5.8</td>
<td>19.3±3.9</td>
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<td>Melittin-stimulated release</td>
<td>75.1±9.6</td>
<td>68.1±15.2</td>
<td>49.9±7.9</td>
<td>23.3±5.1*</td>
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<tr>
<td>Stimulated minus basal</td>
<td>32.7±9.2</td>
<td>40.5±11.4</td>
<td>27.4±4.5</td>
<td>4.0±3.9*</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SEM in nanograms angiotensin I per milliliter per milligram per 30 minutes incubation. Values for stimulated minus basal represent differences from n paired samples; p indicates whether these differences are significant. *p<0.05 compared with all other concentrations by analysis of variance.

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apparent tonic inhibition of renin by EDRF is consistent with this intriguing hypothesis.

We observed that isoproterenol-stimulated renin release was not significantly changed by inhibition of EDRF with either LNMMMA or hemoglobin, although there was an interesting trend for the level of stimulated renin to increase under these conditions. These data would suggest that the basal EDRF produced is not sufficient enough to modify the considerable stimulus for renin release by $10^{-5}$ M isoproterenol. However, when we added the substrate L-arginine to (presumably) amplify EDRF, we found that isoproterenol stimulation of renin release was completely reversed. Thus, it is possible that a "reserve" of substrate could enhance EDRF synthesis, contradicting isoproterenol stimulation. The inactive isomer D-arginine had no effect on renin release. Thus, our combined data support the hypothesis that EDRF can inhibit isoproterenol-stimulated renin release.

The second messenger of endothelium-derived relaxation is cGMP.21 It has been reported previously that atrial natriuretic peptide inhibition of renin in cultured juxtaglomerular cells is mediated by cGMP and calcium independently. Additionally, it has been shown that atrial natriuretic peptide-stimulated cGMP not only inhibits basal renin release but also reverses cAMP-mediated isoproterenol and arachidonic acid-stimulated renin release, implying a modulatory interaction between these two cyclic nucleotides. In adrenal glomerulosa cells, a recently described cGMP-stimulated phosphodiesterase exists that decreases intracellular cAMP downstream from adenylate cyclase.24 If such an enzyme exists in the juxtaglomerular cell, it could account for the apparent sensitivity of cAMP-mediated renin release to increases in cGMP. Our data support the hypothesis that the effect of isoproterenol is particularly sensitive to cGMP-mediated renin inhibition by EDRF. Likewise, our previous finding that pretreatment with acetylcholine (which stimulates EDRF) reversed PGI2-stimulated renin release (also cAMP-mediated) from renal cortical slices7 is consistent with this hypothesis. Thus, although we have not measured cGMP, nor would such measurements from the heterogeneous cell population of the cortical slice be meaningful, our data and the previous work of others21-24 suggest that EDRF-stimulated cGMP in the juxtaglomerular cell is likely the inhibitory second messenger that opposes basal and cAMP-mediated stimulation of renin release.

Previously, we reported that neither of two endothelium-independent nitrovasodilators, nitroprusside and nitroglycerine, mimicked the effect of acetylcholine on PGE2-stimulated renin release.7 However, because at least part of the vasodilation induced by these compounds is attributed to cGMP in the vascular smooth muscle, the lack of any effect undermined our hypothesis that renin inhibition by EDRF was due to this second messenger. Additionally, it has been reported that nitroprusside can inhibit isoproterenol-stimulated but not basal renin release from rat renal cortical slices.23 To address this inconsistency in our previous data and to further support our contention that cGMP can inhibit renin release in vitro, we carried out studies with nitroprusside concentrations at and above those we used previously. We found that not only did nitroprusside tend to decrease basal renin release, but at high concentrations it completely reversed melittin-stimulated renin release. We have previously shown that melittin stimulates renin release by inducing endogenous PG synthesis, reportedly through a cAMP-mediated mechanism.23 Thus, these data further support the hypothesis that increases in juxtaglomerular cell cGMP suppress cAMP-mediated renin secretion. Endothelin is a potent endothelium-derived vasoconstrictor8 that has been reported to affect renin secretion. In vivo, endothelin increases plasma renin activity after lowering renal blood flow and glomerular filtration rate.9 However, in various in vitro preparations, endothelin has been found to inhibit renin release. In isolated glomeruli, endothelin inhibited basal and isoproterenol-stimulated renin release. The calcium channel blocker nifedipine reversed the effect of endothelin.10 Likewise, in dispersed juxtaglomerular cells11 and cortical slices,12 endothelin inhibited renin, and this was reversed in the absence of extracellular calcium.11 These studies suggest that renin inhibition by endothelin is due to channel-mediated influx of the inhibitory second messenger calcium. Additionally, endothelin has been reported to stimulate EDRF production.13 We tested whether blocking EDRF would alter endothelin-mediated renin inhibition or indeed represented a significant component of the response. Our data suggest that inhibition of renin by endothelin is not modified by EDRF and is consistent regardless of what manipulations we
undertake. This supports the previous reports\textsuperscript{10–12} that increased intracellular calcium is the predominant signal in this renin inhibition.

In summary, using pharmacological tools to inhibit or stimulate EDRF, we found that evidence that endogenous renal EDRF can inhibit basal renin release and, when supplemented with substrate, also inhibits stimulated renin release in vitro. Stimulation of renin release by cAMP-mediated agents such as isoproterenol and PGs is inhibited by cGMP-producing agents such as EDHF and nitroprusside, and we suggest there may be a contradicting interaction of these cyclic nucleotides in modulating the release of renin. The vasoconstrictor endothelin also inhibits renin release in vitro, but we did not find EDHF to be a component of this process. The mechanism by which the renin-inhibitory effect of EDHF is integrated into renin regulation in the whole animal remains to be explored.

Acknowledgments

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References


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