Absence of Renin-Like Activity in Rat Aorta and Microvessels

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SUMMARY Vascular renin-like activity was studied in the aortas and the cerebral microvessels of Sprague-Dawley rats and in the aortas of spontaneously hypertensive rats. Methods were employed to maximize detection of tissue renin and to simultaneously minimize contamination of that activity by either plasma renin or nonspecific proteases capable of angiotensin I generation. To this end, renin activity was measured near its pH optimum; plasma renin was eliminated by nephrectomy; and nonspecific proteases such as cathepsin D were either inhibited by proteolytic blockers or removed by chromatography over immobilized bovine hemoglobin. Aortic vascular renin-like activity was detected in rats not subjected to nephrectomy and could be inhibited by preincubation of samples with antimouse renin antibody shown to cross-react and inhibit rat plasma renin activity. Furthermore, vascular renin-like activity disappeared after nephrectomy in parallel with the disappearance of plasma renin activity. In the absence of contaminating enzymatic activities, no tissue renin-like activity could be demonstrated in either aortas or cerebral microvessels of Sprague-Dawley rats or in aortas of spontaneously hypertensive rats. (Hypertension 5: 635-641, 1983)

KEY WORDS • renin • plasma • blood vessels • acid proteases • cathepsin D • spontaneously hypertensive rats

A NUMBER of investigators have proposed that extrarenal vessels may be a source of renin; but measurement of vascular renin (i.e., renin arising in vessel wall) can be complicated by the presence in vessel wall of other enzymatic activities capable of generating angiotensin I (AI) in the renin assay. In the present study, methods were used to maximize detection of vascular renin and to simultaneously minimize contamination of that activity by either nonspecific proteases or plasma renin. To maximize detection of vascular renin, activity was assayed near the pH optimum of renin. Since nonspecific acid proteases can cleave AI from angiotensinogen at near the optimal pH for renin activity, all samples were incubated with proteolytic inhibitors and any residual activity was removed by chromatography over immobilized bovine hemoglobin. Activities of nonspecific proteases were monitored by radiochemical assay. Despite the removal of nonspecific acid proteases, a renin-like activity could be detected in rat aorta. To eliminate the possibility that the renin-like activity in rat aorta represented plasma renin contamination, rats were nephrectomized; and plasma renin activity and vascular renin-like activity were measured at 2, 6, and 24 hours after nephrectomy. After the disappearance of plasma renin activity, no vascular renin-like activity could be detected in aortas collected from either Sprague-Dawley or spontaneously hypertensive rats (SHR). Vascular renin-like activity was also absent from microvessels collected from the rat cerebral cortex.

Materials and Methods

Experiments were designed to examine vascular renin-like activity in both large vessels (aortas) and small resistance vessels (cerebral microvessels). Our preliminary experiments (unpublished observations) as well as experiments reported by others demonstrated the presence of acid proteases in preparations of vascular enzymes. Since acid proteases can cleave angiotensin I from angiotensinogen, methods were used to: 1) remove acid proteases from preparations of vascular renin-like activity; 2) minimize the angiotensin I-generating activity of nonspecific acid proteases; and 3) quantitate any residual proteolytic activity.

In Experiment 1, aortas were collected from seven normal rats for the measurement of vascular renin-like...
activity and proteolytic activity as described below. The effect of a reduction in plasma renin activity on the vascular activity was studied in Experiment 2, in which three subgroups (each with seven nephrectomized rats) were prepared. Aortas were collected from rats either 2 hours (Subgroup 1), 6 hours (Subgroup 2), or 24 hours (Subgroup 3) after bilateral nephrectomy. The response of vascular renin-like activity to hemorrhage, a potent stimulus for renal renin release, was studied in Experiment 3. Six hours after bilateral nephrectomy, each of seven rats was placed in a restraining cage and was bled from its tail vein 15%–20% of its total blood volume (calculated from an estimated total blood volume of 50 ml/kg body weight). After hemorrhage, rats were denied access to fluid. At 24 hours after nephrectomy and 18 hours after hemorrhage, the aortas were collected for study as described below. The possible binding of plasma renin to vessel wall was further studied in Experiment 4 in which aortas were collected from seven normal rats after the standard perfusion and rinses to remove blood (described below). A 20-gauge needle was inserted into the lumen of each excised aorta, and the vessel was vigorously perfused with an additional 50 ml of ice saline. As before, the vessels were processed to measure vascular renin-like activity and proteolytic activity.

In Experiment 5, vascular renin-like activity and proteolytic activity were measured in cerebral microvessels collected from five rats. Since the hypertension of the SHR has been linked to elevated levels of vascular renin, vascular renin-like activity was measured in seven male SHRs (350 g) from the NIH colony (average systolic blood pressure 180 ± 2 mm Hg, mean ± SEM). The SHRs were bilaterally nephrectomized, and 24 hours later the aortas were collected. Renin-like activity and proteolytic activity were measured as in Experiments 1–5. In Experiment 7, the vascular renin-like activity from normal rats was tested against antirenin antibody (described below), and in Experiment 8 the recovery of added porcine renin was determined (as described below).

Diet

In Experiments 1 through 4, male Sprague-Dawley rats (250–350 g) received one dose each of intramuscular furosemide (0.57 mg/kg) and were then placed on a 0.18% sodium diet for 2 weeks. All animals had free access to deionized water. This diet represents only mild sodium restriction and does not interfere with growth. Rats in Experiments 5 and 6 were maintained on the standard NIH diet (0.45% sodium).

Collection of Aortic Samples

Two different anesthetic regimens, either pentobarbital (Abbott Laboratories, North Chicago, Illinois) or ketamine (Parke-Davis, Morris Plains, New Jersey) and acepromazine (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) were used to minimize any effect of anesthetic. No differences in experimental results were noted. After induction of anesthesia, a midline abdominal incision was made; a 20-gauge needle was inserted into the aortic bifurcation, and blood was withdrawn into sterile tubes with potassium-EDTA for PRA determination. After centrifugation, the plasma was separated and rapidly frozen. The chest was opened and an incision made in the right atrium. A 19-gauge butterfly needle was inserted into the left ventricle, and the animal was first exsanguinated and then perfused with 120 ml of ice-cold saline to remove any residual blood. The aorta from the arch to the bifurcation of the iliacs was quickly removed and rinsed in ice-cold saline. The adventitia was carefully dissected free, and its removal was verified by light microscopic examination of several samples. The aorta was cut into rings 1 to 2 mm in length and frozen for later homogenization and chromatographic separation of vascular renin-like activity from proteolytic activity. All tissue and plasma samples were stored in liquid nitrogen.

Affinity Chromatography for Nonspecific Acid Proteases

Chromatography for Samples of Aortas

For each experiment, 1 g of aortas (wet weight) was used. The aortas were frozen and thawed four times. The tissue was suspended in 1 ml of cold column buffer pH 4.0 (0.02 M sodium acetate, 1 mM dipotassium EDTA (Sigma, Chemical Company, St. Louis, Missouri), 1 mM EGTA (Sigma), 1 mM sodium tetra-thionate (ICN Pharmaceuticals, Irvine, California) and 0.25 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma)). Such protease inhibitors have been added to renin during purification and assay procedures and do not inhibit renin activity. The tissue was first homogenized on ice for four 15-second intervals with a polytron homogenizer, and then sonicated with a Kontes microturrisonic cell disrupter (Kontes Corporation, Vineland, New Jersey) for two 15-second intervals. The 40,000 g supernatant was then concentrated to 500 µl in a minicon-B15 concentrator (Amicon Corporation, Danvers, Massachusetts). The molecular weight exclusion of approximately 15,000 was verified with chymotrypsin, pepsin, ovalbumin, and then with porcine renin. The sample was placed on a column of bovine hemoglobin immobilized on Sepharose-4B previously equilibrated with column buffer. The affinity resin was either purchased from Worthington Biochemicals, Freehold, New Jersey, or prepared from cyanogen bromide-activated Sepharose 4B and bovine hemoglobin (twice crystallized, dialyzed, and lyophilized, Sigma). The flow rate through the 0.9 × 15 cm (diameter × length) column was 1.1 ml/hr. Such affinity chromatography was used by Smith and Turk to isolate the acid protease cathepsin D from bovine spleen and thymus. Renin, which has no nonspecific proteolytic activity, passes through the resin with the column buffer. Acid proteases bind to the hemoglobin and can be eluted with a buffer of alkaline pH and high ionic strength (0.1 M Tris, 1.0 M sodium chloride, 1 mM EDTA, and 1 mM EGTA, pH 8.6). Vascular renin-like activity and proteolytic activity were measured in each fraction. The size of the frac-
ions was 1 ml in initial experiments and 2 ml in later experiments.

**Plasma Renin Assay**

Plasma renin was measured by the method of Sealey and Laragh. The AI generated was measured by radioimmunoassay. The difference between the AI generated at 37°C and that present in the sample of 4°C was reported as the plasma renin activity in ng Al/ml/hr.

**Vascular Renin-Like Activity**

Vascular renin-like activity in fractions of the chromatographed samples was measured with nephrectomized rat plasma as a source of angiotensinogen. Homologous plasma was used as the source of angiotensinogen because plasma contains inhibitors of acid proteases that may be removed with purification of substrate. Nephrectomized rat plasma was prepared from blood collected 40 to 48 hours after nephrectomy. The blood was processed rapidly at room temperature to avoid the cryoactivation of prorenin that occurs with prolonged incubation at 4°C blood from anephric donors. The plasma was quickly frozen and stored in liquid nitrogen. The baseline renin activity in each pool of substrate was measured, since it was not practical to prepare one plasma pool of sufficient size for all experiments. All fractions in any single experiment were incubated with aliquots of plasma from the same pool. The angiotensinogen level of each pool was estimated by incubation of the plasma with porcine renin and radioimmunoassay of generated AI. That incubation conditions were sufficient to hydrolyze all the angiotensinogen was verified by the failure of increased concentrations of porcine renin to generate additional AI. No plasma pool whose level of angiotensinogen fell outside of the 95% confidence limits of the mean angiotensinogen level for pooled plasma was used.

Samples were incubated with nephrectomized rat plasma in the presence of dipotassium EDTA, neomycin sulfate, and PMSF. Each sample was individually adjusted to pH 5.9 and divided into two aliquots. One aliquot was incubated at 37°C for 18 hours and the other at 4°C for 18 hours. The 4°C incubation was used as a blank for the endogenous AI immunoreactivity in each sample. In addition, incubations of nephrectomized plasma in the presence of either column buffer or the Tris elution buffer were performed in each experiment and the mean and 95% confidence limits of baseline renin activity were determined. The upper 95% confidence limit for basal renin activity in the nephrectomized rat plasma was calculated for each experiment. Only renin activity that exceeded the upper 95% confidence limit for basal renin activity was reported as vascular renin-like activity. The AI in each incubation was measured by radioimmunoassay. The vascular renin-like activity in each fraction was reported as nanograms of AI per milliliter of sample (ng/ml/18 hrs) per 18 hours, i.e., the length of the incubation used. The immunoreactive AI generated by extracts of vessels after chromatography or by samples of porcine renin after chromatography in the recovery experiments (see below) was serially diluted and measured by radioimmunoassay. The fact that the dilution curves were parallel to the standard curve demonstrated the immunoreactive identity of samples and AI standard.

**Proteolytic Assay**

The proteolytic activity in each fraction was measured as the trichloroacetic acid-soluble peptide fragments of proteolytic cleavage of radioactively labeled hemoglobin substrate. Labeled substrate was obtained either as a gift from Dr. French Anderson (NIH, Bethesda, Maryland) or from New England Nuclear Corporation, Boston, Massachusetts (14C-tagged methylated methemoglobin MEC-728). Bovine spleen cathepsin D (Sigma Chemical Corporation, St. Louis, Missouri) was used to test the sensitivity of the assay. Cathepsin D was serially diluted and aliquots of the dilutions were placed into the proteolytic assay and into the tissue renin assay. The proteolytic assay could detect at least one order of magnitude smaller concentration of cathepsin D than the renin assay could detect by measurement of generated AI.

**Cerebral Microvessel Collection and Preparation**

Cerebral microvessels were prepared by the method of Mrsula et al. Five Sprague-Dawley male rats (Taconic Farms, Germantown, New York) were anesthetized, exsanguinated, perfused with iced saline, and then decapitated. The brains were removed and placed into iced 0.01 M sodium phosphate buffer (pH 6.5) which contained 122 mM sodium chloride, 1 mM di-potassium EDTA (Sigma), 1 mM EGTA (Sigma), 1 mM sodium tetrahydrogen dicarbonate (ICN Pharmaceuticals), 0.25 mM phenylmethylsulfonyl fluoride (Sigma), 0.1% bovine serum albumin (which had been heated in a 1% solution to 60°C for 20 minutes to inactivate proteases), and 0.025% azide. The pia were stripped away and the brains homogenized with 15 vertical strokes in a Teflon glass homogenizer (clearance, 0.15–0.23 mm). The homogenate was centrifuged at 1500 × g for 15 minutes; the pellet was resuspended in homogenization buffer and spun at 1500 × g for 10 minutes. Again the supernatant was discarded. The pellet was resuspended in 0.25 M sucrose, layered over a 1.0–1.5 M sucrose gradient, and centrifuged at 58,000 × g as described. Phase microscopy was used to identify the fraction containing microvessels. Microvessels were then processed and chromatographed in a manner similar to that described for aortas. The column size was 5 × 55 mm.

**Inhibition of Vascular Renin-like Activity by Antirenin Antibody**

In experiment 7, either rabbit antime immunoglobulin antibody (kindly supplied by Dr. Eve Slater, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey) or rabbit nonimmune serum was incubated in a 1:1 dilution with either normal rat plasma or vascular renin-like activity collected after chromatography. The
mixtures were incubated at 4°C for 24 hours. Subsequently, as a source of substrate, nephrectomized rat plasma was added to the mixtures containing vascular renin-like activity. The endogenous angiotensinogen in normal rat plasma provided the substrate for the plasma samples. The AI was measured by radioimmunoassay as noted above.

Recovery of Porcine Renin

In Experiment 8 the losses of activity throughout the procedure were determined by the addition of 0.0075 Goldblatt units of porcine renin (United States Biochemical Corporation, Cleveland, Ohio) to aortas collected from rats 24 hours after bilateral nephrectomy. The mixture was homogenized, chromatographed, and the fractions assayed in the usual manner. The recovery was calculated from the total AI generated in the chromatographed fractions and that present in the initial mixture.

Results

Experiment 1: Aortas from Normal Rats

The affinity chromatogram of 1 g of aortas collected from normal rats revealed two peaks (fig. 1 A). The first peak, which consists of material that did not bind to the column, had AI-generating activity and no proteolytic activity. The combination of renin-like activity in the absence of proteolytic activity was called the "vascular renin-like activity." The second peak, which eluted under conditions of high ionic strength and alkaline pH, contained the acid proteases. When incubated with nephrectomized rat plasma, the acid proteases generated no AI. Although in previous studies\(^1\) and in our own unpublished experiments acid proteases can generate AI from angiotensinogen, in the current study the use of proteolytic inhibitors throughout isolation and assay procedures minimized the capacity of acid proteases to generate AI.

Experiment 2: Aortas from Nephrectomized Rats

By 2 hours after nephrectomy the vascular renin-like activity was 12% of that observed prior to nephrectomy (fig. 1 B). (The ratio of peak areas was calculated by dividing the weights of the peaks plotted on the same scale and cut from paper.) At both 6 hours (data not shown) and 24 hours after nephrectomy (fig. 1 C) no detectable renin-like activity was observed. In all cases proteolytic activity was eluted by the Tris buffer.

The plasma renin activity in normal rats and in rats 2, 6, and 24 hours after nephrectomy is shown in figure 2. The 90% reduction in plasma renin activity by 2 hours after nephrectomy was similar to the 88% reduction in vascular renin-like activity over the same period. At both 6 and 24 hours after nephrectomy the plasma renin activity was near zero, although the activity was well above the limits of detection of the assay.

Experiment 3: Aortas from Nephrectomized, Hemorrhaged Rats

Vascular renin-like activity was measured in nephrectomized rats hemorrhaged 15% to 20% of their total blood volume. The chromatogram, similar to that in figure 1C, demonstrated no renin-like activity.

Experiment 4: Aortas after Additional Washes

When excised aortas were perfused vigorously with additional saline, the renin-like activity in 1 g of aortas was 1.0% (ratio of peak areas) of that observed in Experiment 1.
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Figure 1. Plasma renin activity in normal rats and in rats 2, 4, and 24 hours after bilateral nephrectomy. N = 5, 6, 7, 6 respectively. The error bars represent ± one standard deviation from the mean.

Experiment 5: Microvessels
No detectable renin-like activity was found in cerebral microvessels of the rat brain. Acid proteases were eluted with the Tris buffer in a manner similar to that seen in the other chromatograms of aortic tissue.

Experiment 6: Spontaneously Hypertensive Rats
To remove the source of circulating renin, spontaneously hypertensive rats were nephrectomized. No renin-like activity was found in the aortas of these nephrectomized, genetically hypertensive rats; and the chromatogram resembled that in figure 1C.

Experiment 7: Antibody Inhibition Study
Rat plasma preincubated with nonimmune serum generated 38.7 ± 0.8 ng AI/ml/hr (x ± so), and rat plasma preincubated with antirenin antibody generated no detectable AI. The generation of AI by chromatographed extracts of rat aorta was similarly inhibited and fell from 9.52 ± 0.38 ng AI/min/hr when preincubated with nonimmune serum to 0.45 ± 0.03 ng AI/ml/hr when preincubated with antirenin antibody.

Experiment 8: Recovery of Porcine Renin
The recovery of porcine renin homogenized with rat aorta was measured through two separate chromatographic runs. The overall recoveries were 28.9% and 34.5% (average recovery, 32%).

Discussion
In the present study, tissue renin activity was measured in the aortas and in the cerebral microvessels of rats. Because efforts to maximize renin detection included incubation at acid pH, measures to inhibit and isolate acid proteases were taken. Specifically, proteolytic inhibitors with different mechanisms of action were included in isolation buffers; nephrectomized plasma was used as the source of angiotensinogen because plasma contains inhibitors of acid protease activity;2 acid proteases were physically separated from renin-like activity by affinity chromatography; and a sensitive radiochemical assay was used to demonstrate that renin-like activity was devoid of proteolytic activity. When contaminating activities, arising either from plasma (i.e., plasma renin activity) or from tissue (i.e., acid proteolytic activity), were eliminated, no vascular renin-like activity was detected either in Sprague-Dawley rats or in the spontaneously hypertensive rats whose hypertension has been linked to elevated levels of vascular renin.12-15 Vascular renin-like activity in nephrectomized rats did not increase in response to hemorrhage. Furthermore, the vascular renin-like activity of rats not subjected to nephrectomy could be either reduced by extensive perfusion of the aortic lumen prior to assay or inhibited by preincubation of tissues with antimouse renin antibodies shown to cross-react and inhibit rat plasma renin activity. Overall, no vascular renin-like activity independent of plasma renin was detected.

In the current study, the failure to detect renin-like activity in vessels is consistent with a previous study in microvessels8 but differs from a number of earlier studies1-16 in which investigators observed renin-like activity in the walls of large vessels. In the latter studies,1-16 the activity of proteases was neither measured nor isolated. In most studies, investigators used incubations with pHs below neutrality, a range in which acid proteases can generate AI. Of those that used incubations with pHs above neutrality,13-16 none measured proteolytic activity. Without measurement of proteolytic activity, it is not clear that incubation of tissues at neutral pH obviates the problem of tissue protease contamination. To demonstrate otherwise, neutral proteases present in vascular tissue must be isolated and shown to be free of AI-generating activity. Isolation and assay of neutral tissue proteases may entail the use of different methods and substrates from those used for the isolation and assay of acid proteases. Nevertheless, in the absence of an evaluation of neutral proteases, to assume that tissue protease contamination may be ignored at neutral pH may be unwarranted. An additional
problem may confront studies where renin measurements are made at neutral pH after tissues have been subjected to a low pH. Acidification of plasma to pHs below 4 followed by neutralization activates plasma prorenin to renin.35 Under such circumstances, the prorenin, in plasma contaminating vascular tissue, could contribute to vascular renin-like activity.

One might easily imagine that the renin activity of plasma could also contribute to the measured vascular renin-like activity. Results of the current study as well as results of previous studies3,16 demonstrate a contribution of plasma renin to measured vascular activity. Renin-like activity in aorta appears to arise from the plasma. If renin-like activity arises from plasma, how might one explain the previously reported instances14, 15 in which vascular renin-like activity failed to follow plasma renin activity? If nonparallel changes do not reflect variable levels of contamination by nonspecific proteases, such changes may point to the specific binding of plasma renin to the vessel wall. Vascular renin-like activity could rise or fall not only with plasma renin activity but also with the number and/or affinity of vascular binding sites. In the vessel wall renin could be juxtaposed to angiotensin converting enzyme and to sites of entry to vascular angiotensin II receptors — to effect, possibly, an efficient cascade from angiotensinogen to physiologic effect.

Ideally, one might hope to use the same substrate, a protease known to generate AI from angiotensinogen, to effect, possibly, an efficient cascade from angiotensinogen to physiologic effect. Enzymes and to sites of entry to vascular angiotensin II receptors. From 1 g of tissue our methods recover 32% of the total activity or the activity equivalent to better than 300 mg of tissue — three times the amount of tissue used in previous experiments.3-7, 14

The interpretation of our data relies on nephrectomy to remove the source of circulating renin. We cannot be certain that nephrectomy does not remove some factor(s) other than plasma renin that regulates vascular renin activity. Furthermore, we cannot completely exclude the possibility that some difference in our findings from those of earlier studies results from our use of the soluble fraction of homogenates (which permits removal of acid proteases by chromatography) as opposed to total homogenates (which include membrane components). Even so, many others have reported the presence of vascular renin in preparations without membrane components.1, 8, 11, 15 and membranes have been removed in the purification of renin from a variety of tissues.19-21, 29, 30 It is not likely with our methods that we failed to disrupt cells or intracellular organelles that harbor renin. Indeed, to date no one has convincingly demonstrated a preparation of renin where membrane integrity is essential for activity. It remains possible that renin in the vessel wall could exist in a precursor form (i.e., "prorenin") that we failed to activate in these experiments. However, this seems unlikely.

In conclusion, when contaminating activities (plasma renin and nonspecific proteases) were eliminated, no vascular renin activity was detected in either aortas or cerebral microvessels from rats. These results do not exclude the possible regulatory importance of binding or uptake of renin by vessel wall, but suggest that intramural production of renin in these vessels does not occur.

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