RELATION OF ANGIOTENSIN II TO THE CONTROL OF AUTOREGULATION OF RENAL HEMODYNAMICS IN THE DOG

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ABSTRACT

The relationship between the concentration of angiotensin II in renal hilar lymph and changes in renal hemodynamics in response to reduction of renal perfusion pressure was studied in the dog. Renal blood flow was monitored by an electromagnetic flowmeter and glomerular filtration rate estimated from the clearance of inulin or calculated as the product of the renal plasma flow, plasmacrit and extraction of inulin. Angiotensin II in renal lymph was measured by radioimmunoassay.

In eight of nine animals, the lymph concentration of angiotensin II increased as perfusion pressure was reduced. In six of the eight, pressure reduction was associated with autoregulation of renal blood flow and glomerular filtration rate, indicating vasodilatation of the afferent arteriole. When the perfusion pressure was returned to control levels, the renal resistance increased and lymph angiotensin II concentration fell. In two animals, both the renal resistance and the angiotensin II concentration of the renal lymph increased initially as perfusion pressure was reduced.

In one animal, the concentration of the peptide decreased when perfusion pressure was reduced. This decrease was associated with a drop in renal resistance. However, when perfusion pressure was brought back to control level, the renal resistance increased while the concentration of angiotensin II in renal lymph continued to fall.

The results of this study indicate that the changes in renal resistance associated with alterations of the perfusion pressure are not related to the changes observed in concentration of angiotensin II of renal hilar lymph. Since changes in the concentration of the vasoactive peptide in renal lymph are believed to reflect similar changes within

the renal interstitium, this finding is inconsistent with the concept that angiotensin II mediates the autoregulation of glomerular filtration rate and renal blood flow by controlling afferent resistance. The possible role of the peptide in effecting changes in the distribution of cortical blood flow is discussed.

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by

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INTRODUCTION

In 1925, Ruyter, a Dutch technician, published a description of juxtaglomerular (JG) cells, which he found scattered throughout the cortex of the mouse kidney (71). Ruyter demonstrated that JG cells were present in the walls of the afferent arterioles, and as the name implied, they were located just proximal to the glomerulus. Two years later, Oberling identified JG cells in the human kidney (63). Since then, these cells have been observed in many species including lower vertebrate forms such as fish and amphibia which have an adult mesonephros type of kidney (64,33).

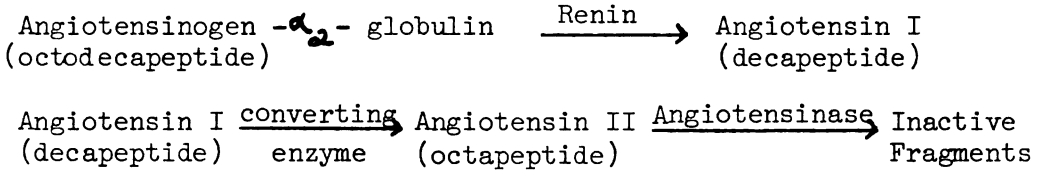
It has been speculated for some time that the kidney has an endocrine function. As early as 1898, Tigerstedt and Bergman demonstrated that crude saline extracts of rabbit kidneys produced vasoconstriction when injected intravenously into rabbits (87). They named the vasoactive substance renin, and by varying their method of preparation, demonstrated that it could be extracted from the renal cortex but not from the medulla. In 1939, Goormaghtigh, on a purely morphological basis, concluded that the JG cells were the source of renin (29). He observed in rabbits, in which the renal arteries were chronically partially occluded, the JG cells contained an increased number of stainable granules. He interpreted this finding as being part of a "glandular cycle" in which chronic stimulation resulted in hypergranulation. Goormaghtigh emphasized that other structures at the vascular pole of the glomerulus might have an important functional relationship with the JG cells. He coined the term "juxtaglomerular apparatus" (JGA). The JGA included, in addition to the JG cells, the polkissen or "lacis" cells situated about the vascular pole of the

glomerulus and the unique group of distal tubular epithelial cells of the macula densa (MD), which come in contact with the JG cells (29,30). In 1943, McManus observed that the Golgi apparatus of the macula densa cells is located in a position quite different from that found in the cells of the rest of the distal tubule (56). Normally, the Golgi bodies are found near the luminal border; however, in the MD, they are located at the base of the cells near the JG cells. Also, McManus demonstrated that the basement membrane between the MD cells and the JG cells was incomplete, allowing intimate contact between the JG cells of the afferent arteriole and the MD cells of the distal tubule.

These early observations in no way proved that the JGA was the source of renin or acted as an endocrine organ. Only within the last ten to fifteen years has direct evidence appeared supporting Goormaghtigh's theory. It has now been well substantiated that the JGA is the source of renin (6,14,15). There is, however, some disagreement as to the exact component within the JGA containing the renin. The major body of evidence points to the JG cell. This evidence includes studies correlating renin content and JG granulation (3,22,24,25,34,36,37,59,88,89,97), studies with fluorescent antirenin antibodies (32,38), and observations on renal cell cultures. The microdissection work of Bing and Kazimierczak indicates that the MD cell is the source of renin (4,5). Bing has hypothesized that renin is formed in the MD cells and stored in the JG cells until released.

Renin itself was not the vasoactive substance in Tigerstedt and Bergman's crude kidney extract. Renin is in fact an enzyme which acts upon a substrate, angiotensinogen, which is formed in the liver, and circulates in the plasma associated with an alpha-2-globulin (43,66,68).

The product of this initial reaction is angiotensin I, a decapeptide which is considered biologically inactive by most investigators. Activation requires the removal of a dipeptide residue from the carboxy end of the amino acid chain. This step is accomplished by angiotensin I converting enzyme. The product of this second step, angiotensin II, is an octapeptide and is considered to be the most potent vasoconstrictor agent known (73,75). Circulating angiotensin II is hydrolyzed to inactive forms by plasma and tissue angiotensinases. The entire reaction is as follows:



Converting enzyme is usually present in the blood; however, the significance of plasma converting enzyme is uncertain. Ng and Vane have shown that the primary site of angiotensin I conversion is probably the lung (62). While plasma conversion appears to be rather sluggish, one passage through the lungs is usually sufficient to convert most of the circulating angiotensin I to angiotensin II. Oparil et al. demonstrated that 50% of infused angiotensin I was converted to angiotensin II by a single passage through dog lung, even when angiotensin I was injected in doses 10,000 times physiological levels (65). The kinetic studies of Huggins et al. indicated that lung converting enzyme is in fact a distinct enzyme and that the rate of conversion by lung is approximately ten times the rate of conversion of plasma (6.7 micromoles/min/mg protein and 0.7 micromoles/min/mg protein, respectively) (41).

Converting enzyme activity has also been demonstrated in extracts from kidney, ileum, liver and heart (11); however, whether this represents specific conversion or nonspecific hydrolysis has not been determined.

Bailie, Rector and Seldin found that renal lymphatic concentrations of angiotensin II were consistently higher than arterial or renal venous plasma concentrations in the dog (1). Also, during hemorrhage, they observed a rise in the lymphatic concentration of angiotensin II before any apparent change in systemic concentration. They infused C^{14}-labeled angiotensin II and measured the specific activity of angiotensin II found in blood and lymph. From this data, they concluded that the high levels of angiotensin II found in renal lymph did not reflect extraction from plasma by the kidney, but represented de novo formation of the peptide within the interstitium of the kidney. Intrarenal formation of angiotensin II was further supported by the work of Horky et al. (39). They demonstrated the presence of renin, angiotensinogen and angiotensin I converting enzyme in the abdominal lymph of rats. Bilateral nephrectomy produced a decrease in angiotensin I converting enzyme activity in both lymph and plasma. Thus, it seems that renin secretion can lead to angiotensin II formation both systemically and intrarenally.

The physiological role or roles of angiotensin II have not been completely established. Early histological observations suggested that JG granulation was in some way linked with sodium balance. In 1955, Hartroft and Hartroft demonstrated that there was a correlation between the degree of JG granulation and the width of the zona glomerulosa in rats placed on high and low salt diets (35,36). It had been observed that adrenalectomy produced hypergranulation of JG cells (3,24).

Davis et al. used elaborate cross circulation experiments to demonstrate a humoral agent released by the kidney stimulated aldosterone secretion (20,21). Later, Genest et al. and Laragh and co-workers reported that synthetic angiotensin II augments aldosterone production in man (26,46). Thus, it appears that angiotensin II in the systemic circulation acts as an aldosterone stimulating hormone. This concept has withstood a great deal of experimental investigation and is at present the only functional role of angiotensin II for which a stimulus, effect and positive feed-back system have been established. The curious anatomical relationships of the components of the juxtaglomerular apparatus have led to a great deal of speculation with regard to a possible intrarenal regulatory role for the renin-angiotensin system. Therefore, a great deal of effort has been concentrated on defining an intrarenal function for angiotensin II and the JGA.

Intravenous injection of angiotensin II or renin into the systemic circulation results in a variety of responses by the kidney. The specific effect in some instances is dependent upon dosage. Soghikian and Lameijer reported that infusion of angiotensin at low rates (0.003-0.1 mg/kg/min) led to antidiuresis and sodium retention while, if the infusion rate was increased (0.06-0.13 mg/kg/min), the result was diuresis and natriuresis (77). This reversal of effect with increasing dosage was not related to changes in blood pressure, because the threshold dosage for the pressor response was 10 to 100 times that required for diuresis (7). Also, it was not related to aldosterone secretion because the time course involved was much too rapid for an aldosterone effect. These effects were apparent within 1-2 minutes after injection. The low dosages associated with the antidiuretic

response caused decreases in sodium excretion, free water clearance, glomerular filtration rate (GFR) and renal blood flow (RBF), while higher dosages caused an increase in sodium excretion and either an increase or decrease in GFR and RBF (7,47). Schroder et al. have shown that the specific effect of angiotensin may be related to the volume and osmolarity of the urine prior to the injection (72). They observed angiotensin and renin lead to a decrease in urine volume during water diuresis and an increase in urine volume during saline diuresis in rats. This is in agreement with the original observations of Pickering and Prinzmetal that the diuretic response to renin injection occurred more often in animals with high control levels of solute excretion (67).

The renal response to angiotensin II can be affected by the rate of adrenal steroid production or the state of sodium balance. For example, angiotensin II treatment in rats usually caused diuresis; however, the response was reduced or reversed by adrenalectomy (18,31). The blood pressure prior to infusion of angiotensin II also has an effect upon the type of response. Usually, hypertension tends to increase the frequency of a diuretic response; however, this relationship is not clear and possibly other determinants might be involved (90).

Thus, it appears that angiotensin infusion can produce changes in renal function. The exact nature of these changes is dependent upon dose, species and sodium balance, as well as other factors. The techniques used for these observations do not pinpoint the exact site or specific action of angiotensin II. The possible intrarenal function of angiotensin II may involve its effects upon tubular sodium transport, GFR, RBF or distribution of renal blood flow.

There is an increasing body of evidence which indicates that angiotensin II may have a direct effect upon the renal tubule. In 1963, Vander used stop flow techniques to demonstrate that angiotensin II infusion caused less sodium to be reabsorbed from the distal part of the nephron (93). However, as he pointed out, this effect could have resulted from an inhibitory action on the sodium transport mechanisms or from permeability changes which would affect passive sodium influx. In birds, the venous return from the legs supplies a large portion of the renal tubules. This unique renal portal system offers an opportunity to study tubular effects of the peptide in relative isolation from arterial glomerular circulation (78,79). The infusion of angiotensin II into the leg vein of birds results in pronounced ipsilateral natriuresis (19,44, 45).

Leyssac has suggested that angiotensin II acts as a mediator in regulating GFR through its effects upon proximal tubular pressure (49, 50,51). He developed a method of estimating proximal tubular reabsorption rates independent of GFR and RBF. This method involved abruptly clamping the renal artery and, by direct observation, determining the time required for the proximal tubules to collapse. He reasoned that the rate of tubular collapse would be directly proportional to the rate of proximal tubular reabsorption. Using this method, he compared proximal reabsorption rates with initial inulin clearances in rats. His data indicated that over the normal range of spontaneous variation in GFR, there was an inverse relationship between GFR and occlusion time. He also found that if angiotensin II was injected prior to clamping, the occlusion time increased. This finding was interpreted as resulting from an inhibition of proximal tubular reabsorption by angiotensin II.

Leyssac pointed out that filtration is in part a function of proximal tubular pressure. He suggested that the angiotensin II concentration in the interstitium affected the rate of proximal sodium reabsorption and thus regulated proximal tubular pressure. In this manner, the peptide functioned as an intrarenal mediator in controlling GFR. In contrast to Leyssac's observations, Wahl, Nagel, Fischback and Thurau found that the occlusion time was constant over a wide range of GFR's (0.3-2.2 ml/min/gm kidney) (98). The reasons for these discrepant findings are not clear.

Lowitz, Stumpe and Ochwadt used micropuncture techniques to study the action of angiotensin II on tubular sodium reabsorption (53). They found that intrarenal arterial peptide infusion of 0.2-0.5 micrograms/min/kg caused a pronounced increase in sodium concentration in fluid collected from the distal tubule as compared to control samples collected with saline infusion alone. However, GFR, sodium excretion and urinary water excretion were not changed with this low dosage. Also, the tubular fluid/plasma (TF/P) inulin ratio remained unchanged. When the infusion was increased to 1.0-1.5 micrograms/min/kg, a similar increase in distal tubular sodium concentration was observed. At this higher rate, urinary water and sodium excretion increased. Also TF/P inulin ratio decreased significantly. They also used the "split oil droplet" method of estimating tubular reabsorption rates. This procedure involves introducing an oil droplet into a renal tubule by micropipet and splitting this droplet with saline. Reabsorption rate is estimated by measuring the change in the length of the saline drop with time. The investigators found that application of angiotensin II to the capillary side of the tubule inhibited reabsorption in the distal

tubule but not in the proximal tubule. They demonstrated that intratubular concentrations of angiotensin II up to 2.5 micrograms/ml had no effect in either the distal or the proximal tubule. This data was interpreted as being inconsistent with Leyssac's hypothesis, since it seems unlikely that, in a dynamic situation, the rate of distal reabsorption could effect proximal tubular pressure.

Thus, at present, much of the *in vitro* data concerning the effect of angiotensin on tubular transport is contradictory. Most results obtained from frog skin, toad skin or toad bladder models have shown no effect of angiotensin II upon sodium transport (2,17). The one exception is the work of McAfee and Locke who demonstrated that angiotensin enhanced sodium transport in the frog skin (54). On the molecular level, Bonting *et al.* demonstrated the peptide had no effect upon the sodium-potassium activated ATPase of kidney homogenates (8).

Some investigators have suggested that angiotensin II acts upon the renal vasculature and that its intrarenal function is related to its effects upon renal hemodynamics. A fall in the GFR of a single nephron can be detected by a decrease in the proximal tubular diameter of that nephron. Thurau and Schnermann used this concept to study the effects of changes in sodium concentration at the MD upon the filtration rate of a single nephron (86). Using micropuncture techniques, they injected lissamine green into a proximal tubule. When the dye reappeared in a distal tubule, the distal segment was punctured and various electrolyte solutions were perfused upstream. The effect upon GFR was ascertained by measuring changes in the diameter of the early proximal tubule. They carried out these experiments on two groups of rats: a "high-renin" group which had been

fed a sodium-free diet to increase JGA renin content, and a "renin-depleted" group which had undergone unilateral nephrectomy and had isotonic saline substituted for its drinking water. They found that with the "high-renin" group, increases in the sodium concentration in the perfusion fluid were associated with a greater frequency of proximal tubular collapse when compared to the "low-renin" animals. They concluded that increases in the sodium concentration at the MD site can lead to decreases in GFR and this effect is related to renin activity.

Cortney, Nagel and Thurau studied the effect of flow rate through the Loop of Henle upon sodium concentration in the early distal tubule (16). Using micropipets, they artificially perfused a single nephron and collected early distal tubule samples. They found that increased perfusion rates were associated with increased distal tubule sodium concentration. They concluded that sodium concentration at the MD is directly related to the GFR.

Thurau has suggested that the sodium concentration at the MD site is the stimulus for a feedback mechanism in which angiotensin II is a mediator in the control of renal hemodynamics. Changes in the sodium concentration at the MD would effect changes in the angiotensin II concentration in the area of the afferent arteriole. The peptide would control the afferent arteriolar resistance thus regulating GFR and RBF (82,83,84).

Brown et al. have shown that the distribution of renin throughout the renal cortex is not uniform (10). The superficial glomeruli have the highest renin content while those located deeper within the cortex are associated with a decreasing renin activity per glomerulus.

These studies were performed on animals with normal salt intake. Horster and Thurau have measured the GFR of single superficial and juxtamedullary glomeruli in rats on normal salt diets (40). They found that the GFR of the superficial nephrons was consistently lower than the GFR of juxtamedullary nephrons. They also demonstrated that if the rats were placed on high salt diets, the superficial nephron GFR increased by 62% while the juxtamedullary nephron GFR decreased. A high salt intake is associated with decreased renin content of the kidney. Since renin is primarily located in the superficial glomeruli, Thurau reasoned that decreased renin content would mainly reflect changes in release of renin by the superficial JGA. A fall in the rate of renin release from these superficial nephrons would result in a decrease in afferent arteriolar resistance and, therefore, an increase in GFR. Thus, the changes observed in the pattern of superficial and juxtamedullary nephron GFR with high salt diet is consistent with Thurau's hypothesis.

The major contradiction to Thurau's theory is the finding that acute reduction of renal arterial pressure leads to increased rather than decreased renin release as detected in renal venous blood (23,76, 96). It has been suggested that renal venous blood may not reflect conditions found in the interstitial fluid bathing the afferent arterioles (9,83,84). The observed increase in renin release might not reflect an increase in interstitial renin activity or an increase in angiotensin II concentration in the fluid bathing the afferent arteriole.

RATIONALE

The present study was undertaken to correlate changes in the renal interstitial concentration of angiotensin II with changes in renal hemodynamics during reductions in renal perfusion pressure. In this way, the function of angiotensin II as a mediator of the control of renal hemodynamics could be more clearly defined.

As previously discussed, recent evidence supports the concept that angiotensin II can be formed within the renal interstitium, and that the concentration of angiotensin II in the renal hilar lymph of dogs reflects interstitial concentration (1,39,42,55). Therefore, angiotensin II was determined in renal hilar lymph before, during and after reductions in renal perfusion pressure in dogs. The changes in lymph peptide concentration were then compared to effects of reduction in perfusion pressure on renal blood flow and glomerular filtration rate.

METHODS

Animal Preparation:

Male mongrel dogs were anesthetized with pentobarbital sodium (30 mg/kg) and artificially ventilated on a respiratory pump (Harvard Apparatus). A catheter was placed in the aorta below the origin of the left renal artery in order to obtain arterial blood samples and monitor blood pressure by strain-gauge transducer (Statham P23 AC). A femoral vein was cannulated for the infusion of an inulin solution and additional anesthetic agent. The left kidney was exposed through an extraperitoneal flank incision and the left ureter cannulated. A polyethylene catheter was passed into the renal vein via the left spermatic vein in order to collect renal venous blood samples. An electromagnetic flowmeter probe (Carolina Instruments Electromagnetic Flowmeter) was placed on the renal artery. A hilar lymph vessel was isolated and cannulated with polyethylene tubing (PE50).

Two techniques were used to reduce renal perfusion pressure. In seven animals, a snare was placed around the aorta above the renal artery, and the pressure was reduced by tightening this snare. In two animals, an adjustable clamp was placed directly on the renal artery. Renal arterial pressure was then monitored via a curved 23-gauge needle attached to a polyethylene catheter and placed directly into the renal artery distal to the clamp.

Approximately two hours prior to starting the experiment, an intravenous infusion of inulin was started at a rate calculated to maintain the arterial plasma level at 25-30 mg/100 ml. A minimum of one hour was allowed for recovery of the animal following completion of surgery.

Experimental Protocol:

Two control samples of blood and lymph were obtained and renal perfusion pressure was then reduced. After a delay of ten to fifteen minutes to allow for equilibrium, one or two additonal collections of blood and lymph were made and the pressure reduced further. Each collection period required fifteen to thirty minutes in order to obtain adequate volumes of lymph. Arterial and renal venous blood samples were drawn at the midpoint of the collection of lymph. Blood was drawn into heparinized syringes and immediately transferred to chilled test tubes containing disodium ethylenediaminetetraacetic acid (EDTA). Lymph was collected in chilled tubes containing EDTA. The volume of lymph varried from 0.3 to 0.5 ml depending on flow rate and length of collection. In some animals, following a stepwise pressure reduction to 50-60 mm Hg, the snare of clamp was released and one or two recovery periods obtained. In other animals, the pressure was reduced to 95-100 mm Hg followed be a recovery period.

Analytical Methods and Data Handling:

Arterial and renal venous plasma and urine were analyzed for inulin using the diphenylamine method of Walser, Davidson and Orloff (99). In three experiments, GFR was estimated from the clearance of inulin in the usual manner. In six animals, GFR was calculated from renal blood flow (RBF), hematocrit (Hct), and the extraction of inulin (Ein); GFR=RBF x (1-Hct) x Ein. To insure accuracy when determining the inulin extractions, protein-free filtrates of each plasma sample were prepared in duplicate, and duplicates of each filtrate were assayed for inulin. Both RBF and RPP were recorded on an Offner Dynograph. Renal resistance (RR) was calculated from the mean renal perfusion pressure (RPP) and total renal blood flow: RR=RPP/RBF (mm Hg/ml/min).

Microhematocrit was determined on arterial blood samples. Sodium in plasma and urine was estimated by internal standard flame photometry (Eppendorf Flame Photometer).

Angiotensin II in plasma and lymph was determined by radioimmunoassay. The procedure used was a modification by Bailie et al. (1) of the method originally described by Gocke and co-workers (27). Antisera were produced in rabbits after immunization with aspartyl-1-valyl-5 angiotensin II (Ciba) conjugated with Freund's adjunctiva as described by Goodfriend, Levine and Fasman (28). Radioiodinated angiotensin II was obtained from Cambridge Nuclear Corp. Tris buffer at pH 8.6 was utilized as it was demonstrated that with this buffer, variations in protein concentration had no effect upon the assay (1). Both supernatant and charcoal were counted in a well-type scintillation counter (Nuclear Chicago). Angiotensin II concentrations in lymph and plasma were expressed in picrograms/milliliter (pg/ml). Details of the procedure are discussed in Appendix I.

RESULTS

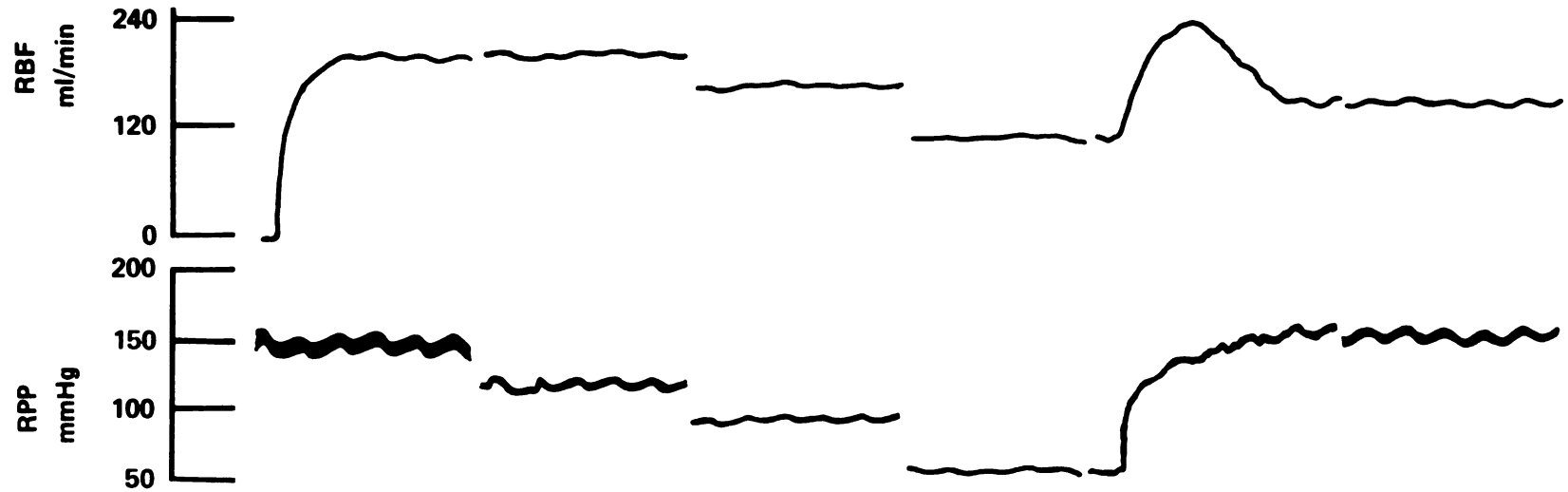
Figs. 1, 2 and 3 show the results of three experiments in which renal perfusion pressure was reduced by tightening a snare on the aorta. Fig. 1 demonstrates results in an animal in which near perfect autoregulation of RBF and GFr was observed. As the perfusion pressure was reduced, RR decreased indicating vasodilatation. The GFR was initially maintained in the face of decreased perfusion pressure, indicating afferent vasodilatation. Each reduction of RPP was associated with an increase in the angiotensin II concentration in the renal lymph: the concentration increased threefold over control. When the snare was released, the renal resistance increased above control and the lymph angiotensin II concentration fell to control levels.

Fig. 2 represents an experiment in which incomplete autoregulation occurred. Renal resistance fell as pressure was reduced but RBF and GFR also declined. GFR was maintained somewhat better than RBF in the initial periods. Lymph angiotensin II rose as the pressure was reduced. When the snare was released, the renal resistance increased and the lymph peptide concentration fell to control levels.

In Fig. 3, incomplete autoregulation of RBF and GFR was initially demonstrated. The snare was then released and a second control period obtained. When the pressure was reduced a second time, RBF and GFR were perfectly autoregulated. In both instances, the lymph angiotensin II increased as pressure was reduced and renal resistance decreased. The final reduction in pressure was associated with an increase in peptide concentration which was almost three times the previous control concentration. When the snare was released, the lymph angiotensin II concentration fell and the renal resistance increased.

FIGURE 1

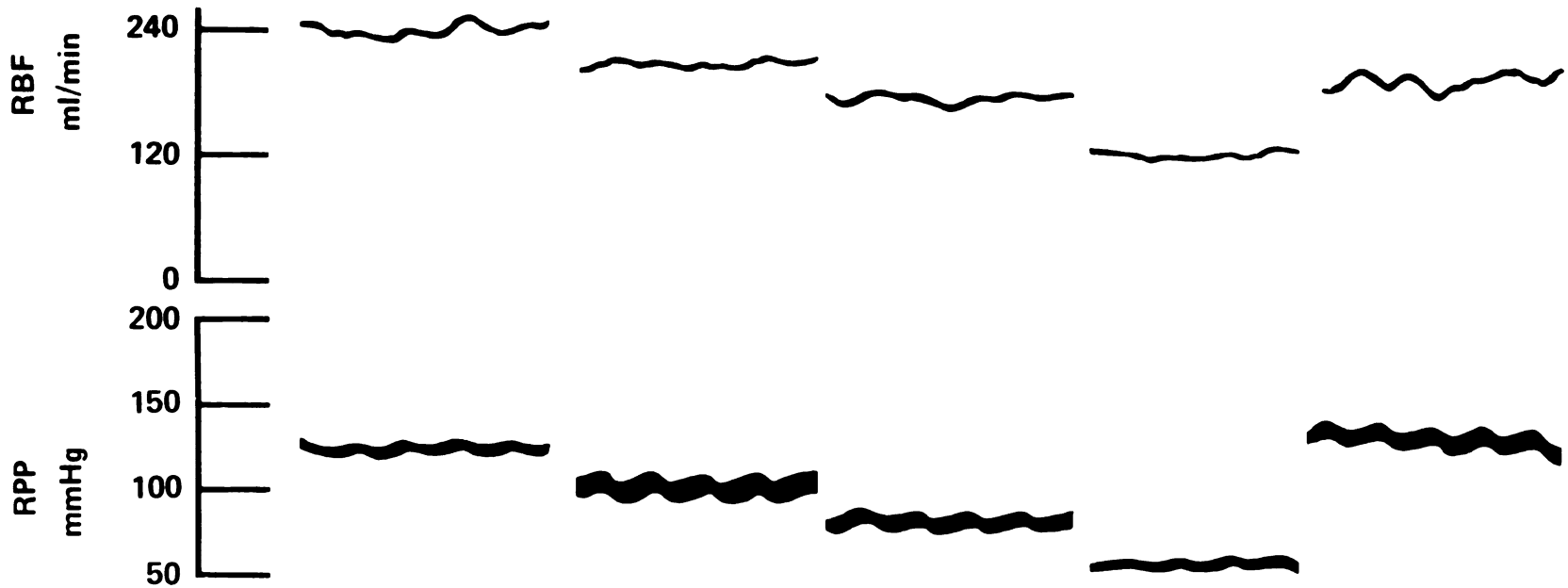
Effect of reduction on renal perfusion pressure (RPP) by an aortic snare on renal blood flow (RBF), glomerular filtration rate (GFR), renal resistance (RR) and renal lymph angiotensin II concentration (Lymph AII).



GFR ml/min/g	0.39	0.39	0.41	0.21	0.43
RR pru	0.70	0.56	0.53	0.50	0.98
AII (LYMPH) pg/ml	131	232	280	395	143

FIGURE 2

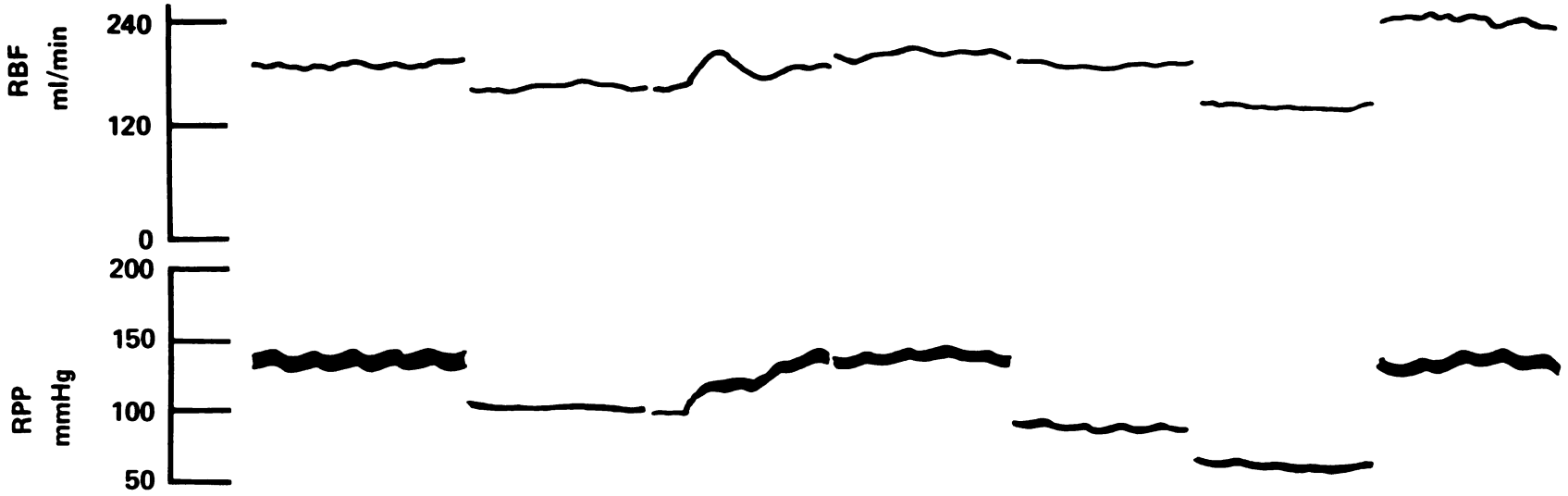
Effect of reduction on renal perfusion pressure (RPP) by an aortic snare on renal blood flow (RBF), glomerular filtration rate (GFR), renal resistance (RR) and renal lymph angiotensin II concentration (Lymph AII).



GFR ml/min/g	0.75	0.73	0.69	0.39	0.68
RR pru	0.52	0.49	0.43	0.47	0.66
AII (LYMPH) pg/ml	347	472	862	862	312

FIGURE 3

Effect of reduction on renal perfusion pressure (RPP) by an aortic snare on renal blood flow (RBF), glomerular filtration rate (GFR), renal resistance (RR) and renal lymph angiotensin II concentration (Lymph AII).



GFR ml/min/g	0.66	0.55		0.64	0.60	1.11
RR pru	0.68	0.58		0.65	0.45	0.44	0.56
AII (LYMPH) pg/ml	140	235		119	200	315	90

Figs. 4 and 5 show the results of two experiments in which the renal perfusion pressure was reduced by a clamp placed directly upon the renal artery. In Fig. 4, the first pressure reduction was associated with an increase in renal resistance and an increase in lymph angiotensin concentration. After the clamp was released, there was a spontaneous decrease in RBF. This change in RBF was associated with an increase in renal resistance and a decrease in lymph peptide concentration. With the second series of pressure reductions, good autoregulation was observed, and as the RR decreased, the lymph concentration of angiotensin II increased fourfold. Fig. 5 represents an experiment in which near perfect autoregulation of RBF and GFR was seen. Again, as pressure was reduced, renal resistance decreased and lymph angiotensin II concentration increased. With the final reduction, the RR appears to have reached a constant value, which indicates that the vasculature was maximally dilated. The lymph angiotensin II concentration had increased threefold at this time. When the clamp was released, the renal resistance increased and lymph angiotensin II concentration fell to half its previous value.

Table I summarizes the data from four additional experiments, in which the pressure was reduced by aortic snare. The second period of experiment B is the only instance in which a decrease in renal resistance is associated with a decrease in lymph angiotensin II concentration. In Experiment D, during the final pressure reduction, the renal resistance actually increased. This was not an autoregulatory response since the vasoconstriction occurred in the face of a decrease in perfusion pressure. In every other instance, a decrease in

FIGURE 4

Effect of reduction of renal perfusion pressure (RPP) by renal artery constriction on renal blood flow (RBF), renal resistance (RR) and renal lymph angiotensin II concentration.

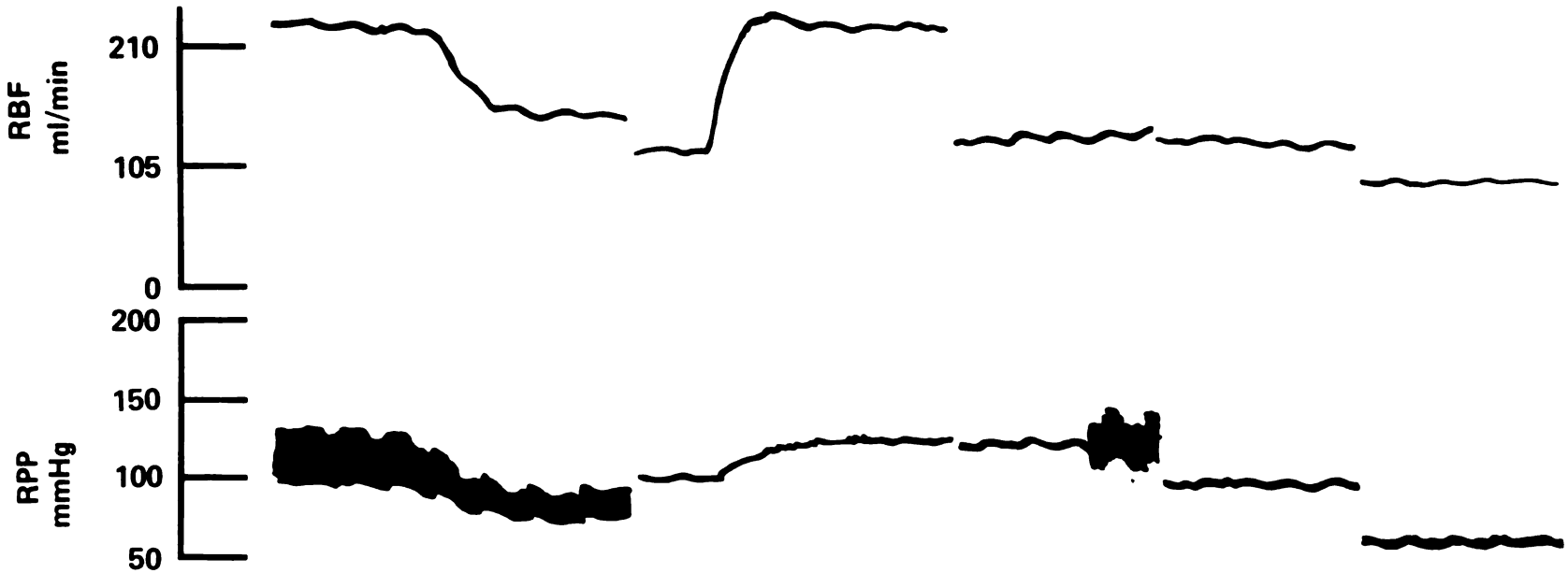

RBF
ml/min
210
105
0
RPP
mmHg
200
150
100
50
RR
pru
0.46
0.53
0.52
0.87
0.71
0.60
AII (LYMPH)
pg/ml
126
220
75
112
366

FIGURE 5

Effect of reduction of renal perfusion pressure (RPP) by renal artery constriction on renal blood flow (RBF), glomerular filtration rate (GFR), renal resistance (RR) and renal lymph angiotensin II concentration.

RBF
ml/min
370
185
0
RPP
mmHg
200
150
100
50
GFR
ml/min/g
0.80
0.85
0.60
0.20
0.57
RR
pru
0.38
0.30
0.23
0.24
0.51
AII (LYMPH)
pg/ml
76
146
180
235
135

TABLE I:

Effect of changes in renal perfusion pressure (RPP) produced with an aortic snare on glomerular filtration rate (GFR), renal blood flow (RBF), renal resistance (RR) and lymph angiotensin II concentration (Lymph AII).

Expt.	RPP	GFR	RBF	RR	Lymph AII
	mmHg	ml/min	ml/min	pru	pg/ml
A	120	35.4	253	0.47	90
	95	31.6	162	0.59	2000
	125	33.4	146	0.86	87
B	116	34.6	203	0.59	1417
	92	31.0	176	0.53	455
	127	34.2	200	0.63	200
C	130	47.5	247	0.53	384
	100	41.7	186	0.53	907
	121	43.5	208	0.59	574
D	150	46.8	411	0.37	161
	130	52.0	400	0.33	245
	100	26.6	280	0.36	477

perfusion pressure was associated with a decrease in renal resistance and an increase in the lymph angiotensin II concentration. When the aortic snare was released, the renal resistance increased and the lymph peptide concentration fell.

In all experiments, the concentration of angiotensin II in hilar lymph was consistently higher than the concentration of the peptide in systemic arterial and renal venous plasma. Table II shows the lymph and plasma concentrations of angiotensin II for the experiments in Table I. Lymph flow rates were determined in four experiments and it was found that changes in the concentration of angiotensin in the lymph were not related to flow rate as previously reported (1). In those experiments in which sodium excretion was measured, it was found that as perfusion pressure was reduced, sodium excretion fell and when the clamp was removed, sodium excretion increased. The one exception to this finding was Experiment B of Table I. In this case, when the pressure was initially reduced, the sodium excretion increased, and rose still further when the snare was released (7.4 to 14 to 24 microequivalents/min).

TABLE II:

Concentration of angiotensin II in renal hilar lymph and arterial and renal venous plasma during changes in renal perfusion pressure (RPP).

Expt.	RPP	Lymph AII	Arterial AII	Renal Venous AII
	mmHg	pg/ml	pg/ml	pg/ml
A	120	90	20	20
	95	2000	97	55
	125	87	45	--
B	116	1417		
	92	455	110	20
	127	200	28	20
C	130	384	20	20
	100	907	60	41
	121	574	40	25
D	150	161	20	20
	130	245	57	40
	100	447	56	45

DISCUSSION

The anatomical relationship of the JGA to the total organization of the nephron has led various investigators to speculate on the functional significance of this structure. The cells of the macula densa are in a unique position: at their luminal border, they are constantly subjected to the conditions of the early distal tubular fluid, while at their base, they are in intimate contact with the JG cells of the afferent arteriole of the same nephron (85). Thus, on a morphological basis alone, there seems to be potential for a feedback mechanism regulating the function of the nephron at the afferent arteriole.

Thurau and his co-workers have proposed that the JGA is involved in regulating RBF and GFR. They have shown that the sodium concentration of early distal tubular fluid is in part a function of flow rate through the tubule (16). Filtration rate could thus affect the sodium concentration in the area of the macula densa. They have suggested that the sodium concentration at the MD acts as the stimulus controlling the release of renin and the formation of angiotensin II. This peptide, through its potent vasoconstrictor action, would then regulate the glomerular filtration rate by controlling the resistance of the afferent arteriole. They have further suggested that autoregulation of renal blood flow would result as a consequence of this mechanism. This would explain the remarkable consistency of RBF and GFR which is observed as perfusion pressure is altered.

One condition which is prerequisite to this theory is that angiotensin II must be formed within the renal interstitium. Specifically, angiotensin II must be generated in adequate concentrations in the area of the afferent arteriole. Angiotensinogen, renin

and angiotensin I converting enzyme have been found in the lymph draining the kidney (39). Also, it has been shown that concentrations of angiotensin II and renin activity in renal lymph are consistently greater than that found in arterial and renal venous plasma (1,48). Extraction studies of labeled angiotensin II indicate that the peptide found in the lymph does not appear to be derived from plasma, but is formed de novo within the kidney (1). In addition, converting enzyme activity has been demonstrated in the single isolated juxtaglomerular apparatus (84). The results of this present study are in agreement with the above observations. The angiotensin II concentrations in renal hilar lymph were consistently greater than the concentrations observed in arterial and renal venous plasma (Table II).

If angiotensin II is a mediator in controlling renal hemodynamics, changes in the interstitial concentration of the peptide should be correlated with the appropriate changes in renal resistance. Afferent vasodilatation would be associated with a decrease in the angiotensin II concentration in the area of the afferent arteriole. It has been shown that various methods of reducing renal perfusion pressure invariably cause an increase rather than a decrease in renal venous plasma renin activity (23,76,96). Thurau has suggested that the changes observed in renal venous blood may not be representative of the conditions in the renal cortical interstitium (84).

Studies on the composition of canine renal hilar lymph by Keyl et al. indicate that hilar lymph is formed in the renal cortex and is unaltered by the medulla (42). The work of McIntosh and Morris on the lymphatics of the kidney and the formation of renal lymph in the sheep is in agreement with this concept. They were able to

identify lymphatics in the cortex and cortico-medullary areas but not from within the medulla. Also, they found that the lymphatic concentration of infused labeled inulin, hippuran and creatinine was similar to renal venous levels. They concluded that hilar lymph appeared to be a modified filtrate of postglomerular blood and that any contribution from medullary regions was negligible (55). Thus, renal hilar lymph should be representative of the interstitial fluid of the renal cortex. Also, the studies indicate that solute in renal lymph is not concentrated by passage through the medulla. Thus, changes in the angiotensin II concentration of renal hilar lymph should reflect changes in the interstitial concentration of the peptide in the renal cortex.

The present experiments demonstrate that with one exception (Table I, Experiment B), the concentration of angiotensin II in renal hilar lymph increased when renal perfusion pressure was reduced. This increase in the peptide concentration was usually associated with some degree of renal vasodilatation. When the perfusion pressure was allowed to return to control levels, the angiotensin II concentration in the lymph fell. Usually, this fall occurred in the face of pronounced vasoconstriction. These findings indicate that changes in lymphatic and thus interstitial concentrations of angiotensin II are not correlated with appropriate changes in renal resistance. It is difficult to reconcile this data with the concept that angiotensin II controls the afferent arteriolar resistance and thus mediates the autoregulation of renal hemodynamics.

Britton has proposed a model which attempts to incorporate Thurau's theory with the observed changes in renin release (9). He has suggested that renin is synthesized at a constant rate within the MD

cells. It is then released either into the afferent arteriole, or into the cytoplasm of the JG cells of the afferent arteriole. Angiotensin II would be formed in the JG cells and exert its effect intracellularly. Systemic renin release and release into the JG cells would be inversely related. Thus, an increase in renal venous plasma renin activity would indicate a decrease in renin release into the JG cell. Since the renin activity and angiotensin II concentration with the JG cells cannot be measured in any functioning state, this theory is difficult to challenge by a direct approach. However, the results of the present study indicate that in some instances (the initial periods of Fig. 4 and Exp. A, B and D of Table I) interstitial concentrations of angiotensin II are not inversely related to renal resistance. Also, it appears that the degree of autoregulation observed is in no way related to the pattern of changes in angiotensin II concentration. Figs. 3 and 4 represent two experiments in which different degrees of autoregulation were observed in the same dog. In each case, the initial series of pressure reductions was associated with poor autoregulation and later, a second series of pressure reductions resulted in improved autoregulation. Both maneuvers were associated with similar changes in the lymphatic angiotensin II concentration. In Fig. 3, the pattern is almost identical.

Any theory regarding the intrarenal function of angiotensin II must take into consideration the type of stimuli associated with the formation of the peptide. Most studies indicate that renin release is increased with a reduction of renal perfusion pressure and with low salt intake (91). Both of these situations would tend to decrease the amount of sodium delivered to the MD site. Thurau's hypothesis assumes that renin release is stimulated by an increase in the concentration of sodium

at the macula densa; however, his speculations are not based upon any direct measurement of angiotensin. Nash et al. have reported that renin release is enhanced by manipulations which decrease the renal sodium load (61). Vander demonstrated that some diuretics caused an increase in renin release when volume depletion is allowed (91,95). High doses of furosemide can increase renin release without salt depletion (60). Vander suggests that high doses might act by directly inhibiting MD sodium transport (94). These studies support the concept that the stimulus for renin release might involve a decrease of sodium flux into the MD cells (61). If the intrarenal function of angiotensin II concerns the effect of the peptide upon the renal vasculature, this function must involve a vasoconstrictive process associated with the above types of stimuli.

Maintenance of GFR during reduction of renal perfusion pressure could involve regulating the degree of efferent vasoconstriction. The overall response of the renal vasculature to renal arterial pressure reduction is vasodilatation. However, the resistance of any particular segment is probably the net result of various neural and humoral influences. Angiotensin II could interfere with the dilatation of the efferent arteriole. As a result, when perfusion pressure is reduced, efferent resistance would decrease less than afferent resistance. This model assumes GFR and RBF are regulated by separate mechanisms, and GFR might be regulated more efficiently than RBF (Fig. 1). The results seen in this study, by themselves, do not contradict this model. However, Leibau et al. demonstrated that reduction of perfusion pressure decreases superficial single nephron GFR to a greater extent than total kidney GFR (52). Also, Horster and Thurau have reported

that in the high renin kidney, juxtamedullary single nephron GFR is approximately 2.4 times superficial single nephron GFR. This relationship is abolished in kidneys which have low renin content (40). Since superficial nephrons are associated with a higher renin content than juxtamedullary nephrons, these observations do not support the concept that angiotensin II mediates GFR by regulating the resistance of the efferent arteriole. Another possibility is that angiotensin II disrupts autoregulation by causing vasoconstriction of either the afferent or efferent arterioles.

The distribution of renin throughout the cortex is not uniform. The JG cells associated with the superficial regions display a significantly higher degree of granulation than those located closer to the juxtamedullary area. Also, the renin content per glomerulus is highest in the outermost layers of the renal cortex and decreases as one approaches the medulla (10). It is conceivable that any stimulus which would cause an increase in angiotensin II formation could consequently create a specific pattern in the concentration of the peptide throughout the cortex. Angiotensin II formation would most likely be greatest in the outermost regions of the cortex. If the peptide influenced the resistance of the renal vasculature, one might expect the blood flow to be shifted toward the inner regions of the cortex.

McNay and Abe have studied changes in the distribution of blood flow in the renal cortex of the dog following reduction of perfusion pressure by aortic clamp (57). Flow distribution was measured by injecting radioactive microspheres into the renal artery and determining the distribution of counts in the superficial and deep regions of the cortex. They found that when the perfusion pressure

was reduced, renal cortical flow was redistributed toward the inner regions of the cortex. Administration of atropine (1 mg/kg) did not alter the results. As stated above, our study indicates that reduction of perfusion pressure by this same maneuver results in an increase in the angiotensin II concentration in renal hilar lymph.

Carriere et al. used the microsphere technique to study the effect of hemorrhagic hypotension upon the intrarenal distribution of blood flow in the dog (13). They reported that hemorrhage also produces redistribution of cortical flow from the superficial region to the deep cortex. Bailie et al. found that hemorrhage increased the concentration of angiotensin II in renal hilar lymph (1). Mild stimulation of the renal nerve also results in this same type of redistribution even when renal blood flow is unaffected (69). Renal nerve stimulation also increases renin release as does the administration of catecholamines (92).

If the distribution of the formation of angiotensin II in the renal cortex is heterogeneous, and if the intrarenal function depends upon this distribution, studies utilizing the infusion of the peptide would be very difficult to interpret. However, it has been reported that if angiotensin I is injected into the renal artery, blood flow distribution is again shifted from the outer regions of the cortex to the juxtamedullary region and total renal blood flow is reduced (12). Since angiotensin I has not been shown to be vasoactive, this effect might be interpreted as resulting from intrarenal conversion (74). It is conceivable that the distribution of converting enzyme activity might parallel that of renin. If so, one would expect that injected angiotensin I would be converted to angiotensin II more efficiently in the superficial portions of the cortex.

Acetylcholine has also been implicated as a factor controlling the intrarenal distribution of blood flow (81). Intrarenal arterial infusion of acetycholine produces an increase in flow through the inner cortical zones and results in a distribution pattern similar to that seen with angiotensin I injection (58,80). However, acetylcholine does not stimulate renin release (91). It should be pointed out that the redistribution associated with acetylcholine infusion appears to be the result of selective dilatation in the inner cortical region whereas the shift associated with angiotensin I infusion involves a selective vasoconstriction in the outer cortical zones (12,58,80). Perhaps both processes could be involved in the redistribution of cortical flow associated with reduced perfusion pressure.

CONCLUSIONS

The angiotensin II concentration in renal hilar lymph increases as renal perfusion pressure is reduced. This is believed to be a reflection of similar changes in the concentration of the peptide within the interstitium of the renal cortex. The renal resistance, as measured by renal blood flow and perfusion pressure, was found not be be related to the changes in the interstitial concentration of angiotensin II. This observation is inconsistent with the concept that angiotensin II mediates the autoregulation of renal blood flow and glomerular filtration rate by controlling afferent resistance.

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APPENDIX I

ANGIOTENSIN II RADIOIMMUNOASSAY

REAGENTS AND MATERIALS

1. 0.1 M Tris Buffer

12.1 gm Trizma base (reagent grade, Sigma Chem. Co.)

1.0 gm Lysozyme (Muramidase) (General Biochemicals)

2.0 gm Neomycin Sulfate (Mann Research Laboratories)

Bring to one liter with distilled water. The pH of this solution should be approximately 8.5. Store in refrigerator.

2. DFP

Diisopropyl Fluorophosphate is diluted 1:20 with isopropyl alcohol and stored in the refrigerator. This reagent is added as approximately 0.01 of volume to pooled plasma and unknown samples just before assay. DFP is extremely toxic. Avoid breathing vapors and avoid contact with skin.

3. Pooled Plasma

A large supply of plasma is dialyzed against 0.003 M EDTA and 0.9 NaCl at 4° C. for 24 hours. This process should remove all preformed angiotensin II. The plasma is then repooled and stored in 3 ml aliquots in plastic vials at -20° C. Before the pooled plasma is used in an assay, DFP is added (1 drop/3 ml) and the plasma is centrifuged to remove the fibrin which forms with storage. Excess pooled plasma is discarded. An aliquot should not be reused.

4. Dextran Coated Charcoal

10% charcoal - 5 gm charcoal (Norit A, Fisher Scientific Co.) Diluted to 50 ml with distilled water.

1% dextran - 1.0 gm dextran (T-70, Pharmacia Uppsala, Sweden). Diluted to 100 ml with distilled water.

Each reagent should be stored in the refrigerator and is good for seven days. Equal volumes of 10% charcoal and 1% dextran are mixed fresh just prior to the separation procedure.

5. I-125 Labeled Angiotensin II

Iodine-125 labeled angiotensin II is obtained at a concentration of 0.1 microgram/ml with a specific activity of approximately 1,000 microcurries/mg (Cambridge Nuclear). This is initially diluted 1:10 with 0.1 M Tris buffer and stored in 0.5 ml aliquots at -20° C. For the assay procedure, one 0.5 ml portion is diluted to 50 ml with 0.1 Tris buffer. This final working solution has a concentration of approximately 5 pg/ml.

The efficiency of the counting process should be such that the final dilution yields approximately 5,000 cpm/0.100 ml. If necessary, the dilution procedure can be modified; however, excess I-125 angiotensin II will decrease the sensitivity of the assay.

The non-specific binding is a useful index for determining the integrity of the isotope-peptide moiety. One minus the NSB should range around 0.90. Any major deviation from this value should be investigated.

6. Angiotensin II Standard

Aspartyl-1-valyl-5 angiotensin II (CIBA) is stored in a concentration of 10 micrograms/ml 0.1 Tris buffer at -20° C. Any concentration less than this is unstable even in the frozen state.

For preparation of the standard for the standard curve, 0.100 ml of the stock solution (10 micrograms/ml) is diluted to 50 ml with 0.1 M Tris buffer. One ml of this solution is then added to four ml of Tris buffer. This final dilution is the 400 pg/0.100 ml STD. The

200, 100, 50, 25, 12.5 and 6.25 pg/0.100 ml STD's are then prepared by serial dilution.

Stock solution	10 ug/ml	
First dilution (1:500)	20 ng/ml	
Second dilution (1:5)	4 ng/ml	400 pg/0.100 ml STD
Serial dilution (1:2)	2 ng/ml	200 pg/0.100 ml STD
(1:2)	1 ng/ml	100 pg/0.100 ml STD
(1:2)	0.5 ng/ml	50 pg/0.100 ml STD
(1:2)	0.25 ng/ml	25 pg/0.100 ml STD
(1:2)	0.12 ng/ml	12.5 pg/0.100 ml STD
(1:2)	0.06 ng/ml	6.2 pg/0.100 ml STD

7. Anti-angiotensin II Antibody

To enhance immunogenicity, synthetic angiotensin II is conjugated with a carrier protein prior to injection. This step involves a carbodiimide (CDI) condensation reaction of the angiotensin II with rabbit serum albumin (RSA).

Condensation Procedure:

10 mg rabbit serum albumin (recrystallized X2, PENTEX)

20 mg aspartyl-1-valyl-5 angiotensin II (lyophilized,(CIBA)

Dissolve in 0.5 ml distilled water

Dissolve 200 mg CDI reagent in 0.25 ml distilled water (1-ethyl-3 [3-dimethylaminopropyl] carbodiimide hydrochloride, Ott Chem.)

Combine the above mixtures, stir at room temperature for 30 minutes.

Dialyze against 7 liters of distilled water at 4° C. for 48 hours. (No. 8 Visling tubing).

Immunization Procedure:

The conjugate of angiotensin II with RSA is emulsified with an equal volume of Freund's complete adjuvant (Difco) by vigorous agitation. The concentration of this final mixture is 5 mg angiotensin II/ml. Chinchilla and New Zealand Albino rabbits (6-7 kg) are immunized by repeated injections at four different sites. Toe pad, intraderma, intramuscular, and intraperitoneal routes are used. Each site is injected with 0.1 ml of the emulsion. The total dose for each immunization is 0.5-0.75 mg angiotensin II. The first five immunizations are performed at intervals of 1 to 2 weeks. Approximately two weeks after the fifth immunization, the animals are bled from an ear vein. Following this, the immunization series can be repeated.

The blood is collected into plastic or siliconized glass containers, since the antibody can be absorbed on the walls of untreated glass. Approximately 50 ml of blood is usually taken with each collection. Topical application of xylene dilates the ear veins and aids in the bleeding procedure. After collection, the blood is allowed to clot and the serum is separated by centrifugation.

Antibody Titration:

All manipulations involving the antibody should be carried out in siliconized glass or plastic tubes.

For use in the radioimmunoassay, the antiserum must be diluted sufficiently to produce approximately 50% binding of the labelled angiotensin II. This essentially sets the zero

point of the standard curve at 50%. The dilution used depends upon the antibody titer of the serum and is a function of the amount of antibody produced by the animal and the affinity of this antibody for the angiotensin II antigen.

To carry out the antibody titration, solutions of the antiserum are made ranging from 1:1,000 to 1:80,000 with 0.1 M Tris buffer. Individual tubes are then set up in duplicate for each dilution as follows:

Control:	0.1 M Tris buffer	0.800 ml
	*pooled plasma	0.100 ml
	I-125 angiotensin II	0.100 ml

Antibody Titration:

0.1 M Tris buffer	0.700 ml
*pooled plasma	0.100 ml
I-125 angiotensin II	0.100 ml
antibody	0.100 ml

*(DFP added)

These tubes are incubated and percent binding is determined as discussed in the procedures section. That dilution of the antibody which will bind 50-60% of the I-125 angiotensin II is used for the immunoassay.

The antibody can be diluted 1:1,000 with 0.1 M Tris buffer and stored in 1 ml aliquots at -20° C. The proper dilutions can then be made just prior to the assay. The antibody should not be stored in dilutions greater than 1:2,500 as these dilutions are unstable, possibly due to absorption on the wall of the container.

SAMPLE COLLECTION

Blood Collection:

Blood should be collected in chilled tubes containing 1 mg EDTA/ml blood. The specimen should be kept on ice and spun down in the cold. The plasma is removed and stored at -20° C. The low temperature retards renin activity. The combination of low temperature and the calcium chelating action of EDTA inhibits converting enzyme and angiotensinase activity.

Lymph Collection:

Lymph samples are collected on ice in chilled containers with approximately 1 mg EDTA/ml lymph to be collected. Tubes may be covered with parafilm to retard evaporation. Lymph samples are stored at -20° C.

ASSAY PROCEDURE

All manipulations are carried out on ice. Frozen samples should be thawed at 4° C. The buffer is used for dilutions and the incubation should be pre-chilled.

Prepare standards, I-125 angiotensin II, and the antibody dilution as described in the reagents and materials section.

The standard curve is set up in duplicate tubes prepared as follows:

Control:	0.1 M Tris buffer	0.800 ml
	*pooled plasma	0.100 ml
	I-125 angiotensin II	0.100 ml

Standards:	0.1 M Tris buffer	0.600 ml
	*pooled plasma	0.100 ml
	I-125 angiotensin II	0.100 ml
	antibody	0.100 ml
	standards (0, 6.25-400 ng)	0.100 ml

0.100 ml of 0.1 M Tris buffer is used for the zero (0) standard.

Duplicate tubes are prepared for each unknown as follows:

Control:	0.1 M Tris buffer	0.800 ml
	*unknown	0.100 ml
	I-125 angiotensin II	0.100 ml
Unknown:	0.1 M Tris buffer	0.700 ml
	*unknown	0.100 ml
	I-125 angiotensin II	0.100 ml
	antibody	0.100 ml

*(DFP added)

The sets of tubes designated as controls are used to calculate the nonspecific binding (NSB). The NSB refers to that portion of the counts which will appear to be bound in the absence of the anti-angiotensin II antibody. This results from either a nonspecific complex formation of the I-125 angiotensin II with proteins or lipid material in the unknown and pooled plasma, or more likely from the dissociation of the I-125 label from the angiotensin II and the absorption of the iodine-125 by similar substances. The NSB is thus a function both of the integrity of the I-125 angiotensin II and of unknown factors in the plasma and lymph samples. Consequently, controls must be run with pooled plasma for the STD curve, an unknown plasma for the plasma samples, an unknown lymph for the lymph samples, and in any other situation in which changes might have occurred within a sample group which would affect the observed NSB.

Incubation:

Mix tubes gently. Allow no drops to remain attached to the walls of the tubes. The tubes are then covered with parafilm and incubated for 18-24 hours at 4° C. The antigen-antibody reaction is heat-labile. Therefore, the mixture must be kept at 4° C. until after the separation procedure.

Separation of the Bound and Free Angiotensin II:

Equal portions of 10% charcoal and 1% dextran are combined as described in the reagents and materials section. Then, 0.100 ml of this dextran-coated charcoal preparation is added to each tube. It is important that the charcoal mixture be stirred constantly during the pipetting since the charcoal will rapidly settle out. Vortex the tubes and centrifuge at 3,000 rpm/15 min at 4° C.

The final step in the separation procedure involves decanting the supernatant from the charcoal button. This procedure can be a very large source of error. Care should be exercised in developing a uniform decanting technique. After the supernatant and charcoal have been separated, the tubes need not be refrigerated.

The charcoal and supernatant are counted in a well-type scintillation counter. We found that the type of tube that was used affected the counting efficiency. Consequently, the charcoal and supernatant should be counted in the same type of container.

CALCULATIONS

Nonspecific Binding:

The nonspecific binding is calculated from each set of control tubes as follows:

$$\frac{\text{cpm supernatant - BG} + \text{cpm charcoal - BG}}{\text{total counts}}$$

(cpm=counts per minute)
(BG=background counts)

(cpm supernatant - BG)/total counts = NSB

Percent Binding:

After the NSB has been determined for each group of samples, the percent binding (%B) can be calculated as follows:

$$\frac{\text{cpm supernatant - BG} + \text{cpm charcoal - BG}}{\text{total counts}}$$

Total counts x (1-NSB) = fraction of the total counts available for binding.

%B = (cpm supernatant - BG)/(total counts x [1-NSB]).

QUANTITATION

A standard curve is constructed by plotting %B (0-60%) against pg angiotensin II standard added (0-400 pg). The angiotensin II concentration of the unknowns is then interpolated from this curve. Since the unknowns are added as 0.100 ml, a dilution factor of 10 will give the final result as pg/ml plasma or lymph.

The standard curve obtained in this assay is not a linear function but rather resembles an exponential curve. Initially, small changes in the amount of cold angiotensin II added produce marked changes in the observed percent binding. However, as more angiotensin II

is added, the effect upon %B becomes less pronounced and the curve approaches zero %B asymptotically. As a result, the sensitivity of the assay depends in part upon the range of values involved. The method is very sensitive for low ranges of angiotensin concentration while samples with values greater than 1-2 ng/ml may need to be diluted.