Investigation of the Presence of "Renopressin" in Kidney Extracts from Rabbits and Rats

BRIAN J. MORRIS, PH.D. AND KENNETH J. ROPER, B.SC. (MED.)

SUMMARY One-kidney, one clip hypertension may be caused by a renal humoral factor that is not renin. In the search for such a factor, Skeggs and coworkers have provided evidence that certain extracts of rabbit kidneys can increase the blood pressure of other rabbits indefinitely by a mechanism having a slow onset. In the present experiments an ethanol extract of rabbit kidneys and an acetone extract of a 2.0–4.0 M ammonium sulfate precipitate were injected into rabbits for 10 to 12 days. By Day 3 the increase in blood pressure (mm Hg) was only 1 ± 3 and 7 ± 2, respectively, but by the end of the injection period, blood pressure had increased by 21 ± 4 (p < 0.025) and 19 ± 4 (p < 0.005). Several weeks later the blood pressure was still significantly elevated above control: 13 ± 2 (p < 0.005) and 15 ± 5 (p < 0.05). A similar pattern of response was seen in unilaterally nephrectomized rabbits. In contrast, injection of an ethanol extract of rat kidneys or a 0.75–2.0 M ammonium sulfate fraction or an acetone extract of the latter had no effect on the blood pressure of rats when injected at comparable doses for 13 to 15 days. The ethanol extract of rabbit kidneys caused a mild increase in the blood pressure of rats, but this was not significant. No increase in blood pressure was seen when renal cells from rats with one-kidney, one clip hypertension were injected intraperitoneally into normal rats. Therefore, although the present experiments support the existence of renopressin in rabbit kidneys, a similar factor could not be demonstrated in extracts of rat kidneys.

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KEY WORDS • kidney extracts • blood pressure • experimental renal hypertension • humoral mechanism • rabbit • rat • renopressin

In the search for the cause of hypertension, much attention has been directed at the kidney. Experimental models of hypertension have been developed by impeding renal function in various ways. Elucidation of the mechanism responsible for the one-kidney, one clip model has proved particularly elusive. Mounting evidence suggests that renin is not the pathogenic factor. However, another unidentified substance of renal origin may be involved. In the search for such a factor, Skeggs and coworkers have carried out a number of innovative experiments over the last few years, which have culminated in the hypothesis that a new factor, which they have termed "renopressin," is responsible for the hypertension in this model.

In their experiments, kidneys of rabbits or hogs were fractionated by acidification, ammonium sulfate, and acetone, and the extracts were injected daily into young rabbits weighing 2.2 to 2.6 kg. Blood pressure rose slowly over the injection period, which was generally 10 days, and remained at an elevated level of 15 to 20 mm Hg above control for months after the injections were stopped. The elevation in blood pressure was not due to renin in the fractions because renin generally produces a rapid increase in blood pressure. Also, there was no correlation between the renin content of the fractions and their hypertension-inducing potency, nor was the hypertension affected by blockade of the renin-angiotensin system using [Sar\(^1\), Ala\(^2\)] angiotensin II. Corticotensin\(^6\) and nephrotensin\(^7\) were also excluded, because they too have an immediate pressor effect. Interestingly, when rabbits with one-kidney, one clip hypertension were immunized actively with hog kidney extracts, some of which had been passed down an antirenin column to remove renin, the hypertension was alleviated substantially within a week or so. Moreover, passive immunization with antibodies to hog kidney extracts reduced blood pressure to normal within a day.

From the Department of Physiology, The University of Sydney, New South Wales, 2006, Australia
Supported by grants from The Life Insurance Medical Research Fund of Australia and New Zealand, and the Children's Assistance Fund.
Address for reprints. Brian J. Morris, Ph.D., Laboratory of Cardiovascular Endocrinology, Department of Physiology, The University of Sydney, Sydney, N.S.W., 2006, Australia
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Furthermore, antibodies to the hog extract were also effective in alleviating completely the hypertension produced by injection of rabbit extract into rabbits. Moreover, a rabbit extract that had hypertension-producing potency in normal rabbits could not increase the blood pressure of one-kidney, one clip hypertensive rabbits, either before or after active immunization. The hypertension-producing activity of rabbit extract was lost by boiling, by incubation at 60°C, by incubation at pH 2 or 10 at 25°C, by incubation of kidneys at 25°C before extraction, by extraction of tissue in the presence of a mixture of 8-quinolinol, phenylmethanesulfonyl fluoride, and EDTA, or by gel chromatography in the absence of 2-mercaptoethanol (Skeggs et al., unpublished results). Renopressin thus has the properties of a protein.

The aim of our present study was to attempt to reproduce the experimental observations of Skeggs and coworkers, who have worked only with rabbits, and then, to explore the possibility that a similar factor may also be present and active in the rat.

A 0.75-2.0 M ammonium sulfate extract was prepared by a method similar to that of Skeggs et al.1 Partially thawed kidneys were homogenized as before and the homogenate was centrifuged at 7,000 g for 5 minutes. The pellet was rehomogenized and recentrifuged, and the supernatants were combined. The pH was adjusted to 1.6 at 0°C and after 10 minutes was readjusted to 6.2. Insoluble material was removed by centrifugation at 20,000 g for 5 minutes. The supernatant was filtered on Whatman No. 1 paper, the pH of the filtrate was adjusted to 4.0, and ammonium sulfate was added to give a 0.75 M solution. After stirring for 10 minutes, this was centrifuged at 20,000 g for 10 minutes, the pellet was discarded, and more ammonium sulfate was added to the supernatant to make it 2.0 M. After further stirring for 10 minutes and recentrifugation, the pellet of precipitated proteins was redissolved in an equal weight by volume of distilled water and dialyzed against distilled water at 4°C overnight. Insoluble material was removed by centrifugation, and the supernatant was diluted with 25 mM sodium phosphate buffer containing 100 mM NaCl, pH 7.2, to give suitable aliquots for injection, and stored at -20°C.

A 2.0-4.0 M ammonium sulfate extract was prepared by taking the supernatant of the 2.0 M ammonium sulfate fractionation step above and adjusting it to 4.0 M in ammonium sulfate. This was then stirred, centrifuged, resuspended, dialyzed, and stored as described above.

An acetone extract of the 2.0-4.0 M ammonium sulfate fraction was prepared by taking the redissolved, dialyzed 2.0-4.0 M fraction above and adding ammonium sulfate to give a 0.6 M solution. The pH was then adjusted to 5.5, and at -10°C an equal volume of acetone, also at -10°C, was added. After centrifugation at 20,000 g for 10 minutes at -10°C, the supernatant was discarded, and the pellet was dissolved in an equal weight by volume of distilled water. This was dialyzed against distilled water overnight at 4°C, and after centrifugation to remove insoluble material the supernatant was mixed with buffer/NaCl solution and stored as before.

Biochemical Measurements

The concentration of protein in the extracts was measured by the method of Lowry et al.,3 using bovine serum albumin (Commonwealth Serum Laboratories, Melbourne) as standard. Renin activity was estimated by its initial velocity of formation of angiotensin I (AI) from sheep angiotensinogen, as described previously.8

Production of One-Kidney One Clip Hypertension

A silver clip with a gap of 0.2 mm was placed on the left renal artery of rats under sodium pentobarbitone anesthesia, and the right kidney was removed. The rats were allowed to recover, and blood pressure was monitored over the succeeding weeks. Half of the rats became hypertensive (systolic blood pressure at 2 weeks = 176 ± 7.6 ± 7.6 mm Hg, n = 7).
Preparation of Renal Cell Suspensions

Rats with one-kidney, one clip hypertension were killed by a blow to the head. The sole remaining kidney was removed, and the cortex was chopped into a slurry with a blade. The tissue was forced gently with a spatula through a stainless steel sieve (60-mesh) with washes of 0.2-0.5 ml medium 199 (Commonwealth Serum Laboratories, Melbourne) and then through a finer sieve (150-mesh). The suspension was injected intraperitoneally using a 19G needle into normal rats so that each rat received cells obtained from one each of the hypertensive rats (mean number of cells injected = 4.3 ± 0.6 SE × 10^7, n = 7).

Measurement of Blood Pressure

Morning systolic blood pressure was measured in conscious, warmed rats by tail cuff plethysmography, using a 3.6 × 2.0 cm cuff, a pneumatic pulse transducer (output = 10–20 mV; Narco Biosystems, Houston, Texas), a pulse discriminator, a Statham P23AA differential pressure transducer, and an Omniscribe EB5237-5 chart recorder (Houston Instruments, Texas). The rat remained calm and unrestrained on the lap of the operator during measurement of blood pressure. Since it has been suggested that such indirect methods of measuring blood pressure should be validated by comparison with direct measurement of central arterial pressure, an appropriate experiment was conducted. Comparison of indirect tail-cuff systolic pressure with direct carotid systolic pressure gave a line of linear regression of slope 1.11 (r = 0.97, p < 0.001, n = 14).

Morning mean blood pressure of prewarmed rabbits was measured directly from the central ear artery while the animals were resting quietly. After injection of local anesthetic (Xylocaine; Astra) a 23G butterfly needle (Abbott, Ireland) was introduced percutaneously into the artery and blood pressure was recorded over 5 minutes, during which time a good pulse was noticed. Mean pressure was calculated as diastolic pressure + 1/3 pulse pressure. The slope of the line of linear regression for comparison of central ear arterial pressure with femoral arterial pressure was 1.03 (r = 0.98, p < 0.001, n = 24).

Statistics

Data were analyzed statistically by Dunnett's t test for repeated measures.

Results

Experiments with Rabbits

Resting mean blood pressure of rabbits, determined several days before the injection period, averaged 60 mm Hg ± 6 SD (n = 20). Their weights averaged 2.2 kg ± 0.07 SE at the beginning, and in each group increased steadily to 2.5 kg ± 0.10 SE by 3 weeks. Blood pressures were read between 0900 and 1300 hours, and injections were given at 1600 hours.

The blood pressure of a group of rabbits injected with the ethanol extract of rabbit kidneys remained close to control levels on Day 3 of the injection period, but on Day 8 was elevated significantly and remained elevated by Day 21, even though injections were stopped on Day 12 (fig. 1a). The renin activity of the

![Figure 1](http://hyper.ahajournals.org/)

Effect of extracts of rabbit kidneys on the blood pressure of rabbits. Extracts were injected subcutaneously daily for 10–12 days, and mean blood pressure was measured at intervals before, during, and after the injection period by inserting a 23 G butterfly needle into the central ear artery under local anesthesia. (a) Rabbits injected with ethanol extract (20 mg protein/day) (n = 5). (b) Rabbits that were unilaterally nephrectomized 6 days before beginning injection of ethanol extract (20 mg protein/day) (n = 5). (c) Rabbits injected with acetone extract of 2.0–4.0 M ammonium sulfate fraction (2 mg protein/day) (n = 5). The mean ± SEM and the outer range of the values recorded is shown. The injection period is indicated by arrows.

* = p < 0.05; ** = p < 0.005.
extract was $2 \times 10^4$ pmoles Al/hr 1/0.1 ml dose. This was apparently insufficient to cause a persistent elevation in blood pressure, as judged by the normalcy of blood pressure on Day 3. Moreover, daily administration of extracts containing 10 Goldblatt units of renin have been shown to have no effect on blood pressure.

Injection of the ethanol extract was repeated in a group of unilaterally nephrectomized rabbits (fig. 1b). A similar response in blood pressure was observed: blood pressure was close to normal on Day 3, but by Day 7 was elevated significantly and remained high after the injection period.

The acetone extract of the 2.0–4.0 M ammonium sulfate fraction of rabbit kidneys also raised blood pressure (fig. 1c). Blood pressure was slightly higher than normal on Day 3, but by Day 7 blood pressure had increased further and remained elevated after the injection period. Renin activity of the extract was 620 pmoles Al/hr 1/0.1 ml dose.

In each of the above experiments, 4/5 of the rabbits attained blood pressures that were consistently 2 standard deviations above mean control blood pressure. In a fourth group of rabbits that were injected with 20 mg of bovine serum albumen/1.0 ml 0.9% NaCl for 10 days.

**Figure 2.** Effect of extracts of rat kidneys on the blood pressure of rats. Extracts were injected intraperitoneally daily for 13–15 days and systolic blood pressure was measured at intervals before, during, and after the injection period by tail cuff plethysmography. (a) Rats injected with ethanol extract (1 mg protein/day) (n = 8). (b) Rats injected with 0.75–2.0 M ammonium sulfate fraction (3 mg protein/day) (n = 10). (c) Rats injected with 2.0–4.0 M ammonium sulfate fraction (1.5 mg protein/day) (n = 10). (d) Rats injected with acetone extract of 2.0–4.0 M ammonium sulfate fraction (0.5 mg protein/day) (n = 8). (e) Rats that had been unilaterally nephrectomized 13 days before injection of acetone extract of 2.0–4.0 M ammonium sulfate fraction (0.5 mg protein/day) (n = 8).

* = p < 0.05.
days, blood pressure remained at control levels for at least 3 weeks (Δ = + 2 ± 4 SE mm Hg, n = 6).

Experiments with Rats

The average resting systolic blood pressure of rats in the control period of 1 week before the injection period was 120 mm Hg ± 10 SD (n = 101). No significant increase in blood pressure occurred in groups of rats injected with either the ethanol extract, the 0.75-2.0 M ammonium sulfate extract, the 2.0-4.0 M ammonium sulfate extract, or the acetone extract of the 2.0-4.0 M ammonium sulfate fraction of rat kidneys (fig. 2). Renin activity of the four extracts, expressed as pmole AI/hr/ml per daily dose was 3400/0.05 ml, 8400/1.0 ml, 2500/1.0 ml, and 120/1.0 ml, respectively.

A mild, but not significant, increase in blood pressure was noticed after injection of the ethanol extract of rabbit kidneys into rats (fig. 3). A similar mild increase was observed when the same extract was injected into unilaterally nephrectomized rats, but this achieved statistical significance only on the 15th day after the injections had been stopped.

Transfer of renal cells from one-kidney, one clip hypertensive rats into the peritoneal cavity of normal rats had no significant effect on their blood pressure (fig. 4).

Discussion

The present experiments support the hypothesis that rabbit renal cortex contains a factor capable of producing chronic, sustained, mild elevations in the blood pressure of rabbits by an effect requiring several days before blood pressure begins to rise. An acetone extract obtained after ammonium sulfate fractionation was reported to be most effective. Doses of extracts containing 1, 5, 21, and 53 mg protein were equally potent in raising systolic blood pressure of groups of three rabbits. The average maximum increase in blood pressure recorded during the experiment ranged from 23 to 33 mm Hg, and at the end of the experiment was 15 to 18 mm Hg above control. In the present experiments, a dose of 2 mg protein of acetone extract of a 2.0-4.0 M ammonium sulfate precipitate increased mean blood pressure 19 mm Hg ± 4 SE (n = 5) and at the end of the experiment blood pressure was 15 mm Hg ± 5 SE above control. These changes in mean blood pressure would appear to be similar to the changes in systolic blood pressure measured by Skeggs et al. using a Grant-Rothschild ear capsule. Moreover, the time required to reach maximum blood pressure was similar: 9-15 days vs 7-13 in the present experiments.

An ethanol extract, prepared by an unpublished method of Skeggs, was similarly effective in increasing blood pressure to a maximum of 21 mm Hg ± 4 SE above control. Unilateral nephrectomy before injection had little effect on the pattern of response or the increase in blood pressure achieved (26 mm Hg ± 3 SE), indicating that the presence of both kidneys was not required for the ethanol extract to work and that removing one kidney did not increase the sensitivity of the rabbit.

The properties of the factor in the extracts responsible for increasing blood pressure resemble those of

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** Effect of extract of rabbit kidneys on the blood pressure of rats. (a) Rats injected with ethanol extract (2 mg protein/day) (n = 8). (b) Rats that had been unilaterally nephrectomized 7 days before injection of ethanol extract (2 mg protein/day) (n = 8).

* = p < 0.025.
a protein. However, identification of the protein, termed "renopressin," will be difficult because of the length of the bioassay for it. Since the 4M urea eluate from a pepstatin affinity column was active renopressin may have something in common with the active site of carboxyl proteinases. Although renin is a carboxyl proteinase, it has been excluded. Interestingly, inactive renin does not bind to pepstatin, but does bind to antirenin.14

The mechanism by which renopressin increases blood pressure is also unknown. However, results of experiments thus far offer several clues. Even though its effect on blood pressure is slow in onset, antibodies to it will restore blood pressure to normal within a day.6 The results of experiments with antibodies show that it is renopressin itself that is maintaining blood pressure at least by 2 weeks after the period of injection. It would be unusual for renopressin to have a metabolic clearance rate low enough to account for such a result. Perhaps the antibody is not neutralizing residual injected renopressin, but rather endogenous renopressin released by the rabbit's own kidneys, as a direct or indirect response to the injection, to set up a positive feedback effect for maintaining blood pressure at an elevated level indefinitely. In this respect, it is interesting to note that hyperplasia of the juxtaglomerular apparatus was reported in rabbits injected with kidney extract,5 so that renopressin may possibly originate from the same cells that secrete renin. It is also interesting to note that renopressin could not increase blood pressure by more than about half the increase in blood pressure seen in one-kidney, one clip hypertensive rabbits, even though experiments with antibodies suggest that it is responsible for all of the increase in blood pressure in rabbits with one-kidney, one clip hypertension. The present experiments indicate that injection of renopressin increases blood pressure similarly in rabbits with one or both kidneys, so that the higher pressures seen in the one-kidney, one clip model would appear to be related to effects of the clip.

For renopressin to have general relevance in research on the cause of one-kidney, one clip hypertension, it is important that such a factor be demonstrated in other species. Although extracts from rabbit kidneys were effective, similar extracts from rat kidneys had no hypertension-inducing potency in rats, despite being administered in doses 3–15 times higher than those found to be effective in rabbits. Therefore, it is possible that rat kidney does not have a renopressin. Alternatively, a rat renopressin may differ physicochemically to such an extent that it was not present in the final extracts, or a rat renopressin may be more labile, or it may not be physiologically active after extraction, or it may work differently in vivo or rat kidney may contain a higher activity of inactivating enzymes or factors.

Interestingly, a mild elevation in blood pressure was observed when rats were injected with the ethanol extract of rabbit kidneys. Although the increase was not significant for the group, a small proportion of rats did exhibit hypertensive levels of blood pressure after the injection period. This suggests that active rabbit extract may also be partially effective in rats.

The fact that renal cells from rats with one-kidney, one clip hypertension could not increase the blood pressure of recipient rats may reflect a lack of a humoral factor in the kidneys of the hypertensive rats or the necessity for altered renal hemodynamics, due to the constricting renal artery clip, for a "renopressin" to be released.

Since active immunization of one-kidney, one clip hypertensive rabbits with rat tonin has been shown recently to lower the blood pressure of those rabbits that developed tonin antibodies,16 it may be that renopressin is related to tonin. A fall in blood pressure has also been observed following administration of antiserum to rat tonin to rats with one-kidney, one clip hypertension.16 Interpretation of all such experiments, however, needs to be made with caution as nonspecific effects of the antisera may also be involved. The fact that tonin activity was elevated in the submandibular glands of uninephrectomized hypertensive or normotensive rats suggests an involvement of a submandibular pressor mechanism, at least in the rat.
Whether there might be a species difference in the organ that produces renopressin to cause hypertension is not clear. However, it is interesting to note that tonin did not increase the blood pressure of normotensive rats, an effect also observed in the present study with extracts of rat kidneys. In any case, two observations point to a potential role of tonin in this model of hypertension, namely, the observation that tonin can potentiate the action of norepinephrine and cause direct vasconstriction in the rat mesenteric preparation by a mechanism that could not be blocked by an angiotensin antagonist, and the recent report that tonin can increase Ca\(^{2+}\) in the vascular smooth muscle cells of aortic strips from rats with one-kidney, one-clip hypertension. Further work is required to establish the relationship, if any, between renopressin and tonin.

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