Increased Levels of New Spasmogenic Substances Released by Trypsin from Plasma of Hypertensive Rats

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SUMMARY  The present study was undertaken to evaluate the participation of the kallikrein-kinin system in the normalization of blood pressure after release of the clip in one-kidney, one clip hypertensive rats (1K1C). Kininogen was determined before and after unclipping by tryptic digestion of denatured rat plasma, and spasmogenic activity was measured with isolated guinea pig ileum. In contrast to human plasma for which bradykinin (BK) is the only trypsin-releasable spasmogenic substance (TRSS), rat plasma contains non-BK TRSS (Fractions P₁ and P₂) as well as BK. Fractions P₁ and P₂ were separated from BK by SP-Sephadex chromatography. An increase of total TRSS was demonstrated 60 days after clipping and reached a maximum at approximately Day 75, which was two times that of the normotensive controls (NC). The level of total TRSS did not change after unclipping. The increased level of TRSS in the hypertensive state confirmed the observations of other investigators who reported increased kininogen levels but who could not distinguish between BK and non-BK TRSS because bioassays were performed without prior chromatographic separation of the spasmogenic activities. Fractions P₁ and P₂ were present in the TRSS of both 1K1C and NC plasma, but were two to six times higher in 1K1C and thus probably accounted quantitatively for the increased TRSS in 1K1C. The data suggest that in the hypertensive state there is an alteration in the relative amounts of some plasma proteins that contain non-BK TRSS within their amino acid sequences. Fractions P₁ and P₂ also contain potentiating peptides and have not yet been purified to homogeneity. Pharmacological, physiological, and biochemical data were used to distinguish P₁ and P₂ from BK, BK-homologs having Lys before Arg of BK, the angiotensins, and other substances described in plasma or tryptic digests of the plasma of hypertensive animals. Fraction P₁ increased the blood pressure of awake normotensive rats and was activated by chymotrypsin, whereas P₂ was hypotensive, with intravenous and intraaortic injections being equipotent. The relationship to T-kinin, Ile-Ser-BK, reported to be present in tryptic digests of rat plasma after this paper was submitted for publication, remains to be determined. (Hypertension 6: 255-261, 1984)

KEY WORDS • renal hypertension • kallikrein-kinin system • plasma trypsin-releasable spasmogenic substances • plasma kininogen of hypertensive rats

THERE is some evidence that during the first hours after removal of renal artery constriction a vasodilator mechanism occurs that contributes to the process of normalizing pressure.¹ ² Since one-kidney, one-clip hypertensive rats (1K1C) show a remarkable vascular reactivity to bradykinin (BK),³ this peptide may be considered a likely candidate for the humorally mediated vasodilation that occurs after unclipping. Total plasma kininogen would be expected to be reduced if BK were rapidly mobi-

lized, provided that the rates of kininogen entry into, and disappearance from, blood and kininase activity were not significantly modified.

Our original objective was to measure total plasma kininogen levels of 1K1C rats by the method of Diniz and Carvalho⁴ before and after the release of the renal artery clip. This indirect method utilizes extensive trypsin hydrolysis at pH 7.8 of acid-denatured plasma. The biological activity of the trypsin-released spasmogenic substance(s), denoted TRSS in this paper, is measured with the isolated guinea pig ileum and expressed as BK equivalents. The release of spasmogenic substances other than BK and the release of BK-potentiating peptides⁵ ⁶ are the two major sources of error that could affect the specificity and quantitation of the measurement.

We recently validated this method for the determination of total human plasma kininogen by chromatographic methods that demonstrated that BK was in fact the only TRSS detected in the ethanol soluble fraction of the incubation mixture.⁷ Furthermore, since BK was...
also separated from potentiating peptides by SP-Sephadex chromatography, the measurement of BK in the column effluent by bioassay provided a reliable determination of BK and hence total human plasma kininogen. Indeed, the quantitative chromatographic data agree well with values obtained by radioimmunoassay of tryptic digests of human plasma.

In our present study, we applied the same chromatographic procedures to rat plasma TRSS. We found that rat plasma, in contrast to human plasma, contains TRSS that can be distinguished from BK. Moreover, the non-BK TRSS was about twofold higher in conscious unrestrained 1K1C than in normotensive control (NC) rats, suggesting an alteration in the relative amounts of at least some rat plasma proteins in the renal hypertensive state. Furthermore, the level of rat plasma TRSS did not change after unclipping when the pressure had normalized.

Materials and Methods

The following materials were used: SP-Sephadex G25 and Sephadex G-25-F (Pharmacia Fine Chemicals, Piscataway, New Jersey), AG11-A8 ion-retardation resin (Bio-Rad Laboratories, Richmond, California), trypsin (DPCC), chymotrypsin (CDI), and carboxypeptidase B (COBDFP) (Worthington Biochemicals, Freehold, New Jersey). Endo-oligopeptidase A was provided by Professor A.C.M. Camargo, Faculty of Medicine of Ribeirão Preto, USP. Bradykinin, angiotensin I, II, tetradecapeptide, and BPP (Glu-Lys-Trp-Ala-Pro) were prepared by Professors A.C.M. Paiva and L. Juliano, Escola Paulista de Medicina, São Paulo, Brazil. All other chemicals were reagent grade or equivalent.

One-Kidney, One Clip Goldblatt Hypertension

Male Wistar rats weighing 200 to 300 g were used. One-kidney, one clip hypertension was produced by constriction of the left renal artery with a silver clip as described by Schaffenburg, with simultaneous right nephrectomy. Blood pressure was measured periodically by the tail phethysmographic method. Only animals that exhibited blood pressure levels above 160 mm Hg were used (normal, 110–120 mm Hg). Hypertensive rats were used 1 to 3 months after applying the clip to the left renal artery when the hypertension was stable for at least 2 to 3 weeks.

Direct blood pressure was measured in unanesthetized 1K1C and NC by means of a plastic cannula inserted into the abdominal aorta through the femoral artery and connected to a Statham P.23 transducer and to a recorder (Hewlett-Packard Model 7858A). Blood was collected from the descending aorta through a cannula inserted into the left carotid artery with a plastic syringe containing sodium citrate (2 ml/g). Both cannulas were implanted under ether anesthesia and exteriorized through the dorsum of the rat. We allowed 24 hours to elapse before measuring blood pressure and obtaining blood for the following experiments in different groups of rats: 1) analytical determination of plasma TRSS of 1K1C 2 to 4 months after clipping (Figure 1); 2) analytical determination of the effect of unclipping on plasma TRSS (Figure 2); preparative experiments for the characterization of non-BK plasma TRSS in 1K1C and NC (Figures 3 and 4).

Blood pressure was measured directly, and then 0.5 ml blood was removed from animals placed on a small platform (10 X 20 X 30 cm high) where they remained unrestrained and usually quiet after an initial period of exploratory movements. For the unclipping experiment (Experiment 2), the clip was removed from 1K1C under light anesthesia, as described elsewhere, and blood pressure measurements and blood removal were done on the conscious animals at 0, 2, 6, and 24 hours after clip removal, as described above. The NC rats underwent the same surgical procedures. To assess the effect of unilateral nephrectomy on plasma TRSS, right nephrectomized rats were studied 2 to 3 months after surgery.

Analytical Determination of TRSS

Plasma was obtained by centrifugation at 3000 g for 15 minutes at 23°C. After 1:10 dilution (vol/vol) with 0.2% acetic acid, the solution was heated to 100°C for 30 minutes, neutralized to pH 7.8, and incubated with trypsin (1.0 mg/ml plasma) at 37°C. TRSS activity was measured by bioassay on the isolated guinea pig ileum and expressed as BK equivalents. Plasma samples had spasmogenic activity only after trypsin treatment.

Bioassays

Isolated Guinea Pig Ileum

The bioassay of spasmogenic material was performed by measuring the isotonic contraction of the isolated guinea pig ileum in the presence of benadryl and atropine. Effluent from the SP-Sephadex column was added directly to the bath, and effluent containing 5% acetic acid from the Sephadex G-25 column was lyophilized and redissolved in water before assay. When samples contained trypsin or chymotrypsin, both of which sensitize the ileum to BK, the tissue was repeatedly pretreated with the appropriate enzyme (4 μg/ml organ bath) until the response to BK was constant. Endo-oligopeptidase A did not modify the sensitivity of the ileum to BK or TRSS from rat plasma.

Potentiation of BK was measured with the guinea pig ileum. The test solution was added to the bath 1 minute before adding BK (1 X), and the contraction was compared to that obtained with the same dose of BK alone. One unit of potentiating activity is the amount of test solution required to increase the response to 1 X BK to that of 2 X BK. Samples containing spasmogenic activity (ethanol extracts of the tryptic hydrolysate, column effluent, P, or P) were prediluted to the level where they did not contract the ileum when added to the bath before the addition of BK.
To determine if spasmogenic substances were potentiated by BPP, the potentiating peptide was added to the bath at a concentration of 1 μg/ml 1 minute before the addition of the spasmogenic substances (trypsin hydrolysate, column effluent, P₂, P₃, or known peptides such as BK). The same tissue fragment was used for each pair of experiments (1K1C, NC) to measure spasmogenic or potentiating activity.

The spasmogenic activity of TRSS on the isolated guinea pig ileum was stable for several months when stored at -22°C in the SP-Sephadex chromatography buffer, in 5% acetic acid, or after lyophilization of acetic acid and resuspension in water.

Rat Duodenum and Uterus

The effect of P₁, P₂, and P₃ on isolated rat duodenum and uterus (contraction or relaxation) was measured as described for the guinea pig ileum except that Jalon solution was used.

Other Tissues

The laminar superfusion technique was used to determine the activity of P₁, P₂, and P₃ on the terminal portion of the cat jejunum, cat ileum, and rat colon. The tissue was immersed in mineral oil and superfused with Krebs solution containing 20 μg/ml hioscine hydrobromide, 20 μg/ml phenyloxybenzamine, 400 μg/ml propranolol hydrochloride, 40 μg/ml methysergide bimaleate, and 1 μg/ml indomethacin.

Incubation with Proteolytic Enzymes

Ethanol extracts containing TRSS or fractions prepared by chromatography were incubated with chymotrypsin (25 μg/ml), trypsin (100 μg/ml), carboxypeptidase B (0.6 μg/ml), and endo-oligopeptidase A (2.5 to 5.0 μg/ml) in 0.05 M Tris-HCl buffer, pH 7.7, containing 0.06 M NaCl, 6 ml/hour. The sample corresponded to 6 to 15 ml plasma (3 to 8 ml was applied to the column). The column was eluted with deionized water at 10 ml/hour, and fractions of 2.0 ml were collected. The TRSS present in the effluent was detected with the isolated guinea pig ileum, and the salt-free solutions were lyophilized before chromatography on SP-Sephadex.

Chromatography on SP-Sephadex

The equilibrium chromatography system described by Sampaio et al. was used to separate BK from potentiating peptides that were also released from human plasma by trypsin. The material obtained after desalting on AG11-A8 resin (0.9 x 200 cm) as described by Reis et al. The sample corresponded to 6 to 15 ml plasma (3 to 8 ml was applied to the column). The column was eluted with deionized water at 10 ml/hour, and fractions of 2.0 ml were collected. The TRSS present in the effluent was detected with the isolated guinea pig ileum, and the salt-free solutions were lyophilized before chromatography on SP-Sephadex.

Desalting on AG11-A8 Ion Retardation Resin

The ethanol-soluble extract was desalted by chromatography on AG11-A8 resin (0.9 x 200 cm) as described by Reis et al. The material obtained after desalting on AG11-A8 was dissolved in 0.05 M MOPS (3-[N-morpholino]-propanesulfonic acid) buffer, pH 7.0, containing 15% polyethylene glycol. The column was equilibrated and developed with 0.02 M Tris-HCl buffer, pH 7.7, containing 0.06 M NaCl, 6 ml/hour. The experimental details are given in reference 7 and in the legend to Figure 3.

Chromatography of P₁ and P₂ on Sephadex G-25 Fine

The column (0.9 x 250 cm) was equilibrated and developed with 5% acetic acid, pH 2.7, 12 ml/hour at 23°C. Fractions P₁ plus P₂ (Figure 3) were combined, lyophilized, dissolved in 5% acetic acid and acidified with glacial acetic acid to pH 2.7 before application to the column.

Protein Determination

The method of Lowry et al. was used to measure plasma protein using bovine serum albumin as the standard.

Statistical Analysis

Student’s t test was used to compare average values and differences were considered significant at p < 0.05.

Results

Plasma TRSS Activity of RHR before and after Clip Removal

Plasma TRSS activity of 1K1C (Figure 1 B) reached a maximum on the 75th day (13.5 ± 1.2 μg/ml);
it was about threefold higher than that observed on Day 40 (3.68 ± 1.4 μg/ml). By Day 120, TRSS was markedly reduced to near the level observed on Day 40. In contrast to TRSS, the level of hypertension exhibited by the rats was uniform during the entire period. The level of the TRSS of NC measured during the same period was 4.1 ± 0.25 μg/ml and that of the uninephrectomized controls (n = 4) was 1.5 to 2.0 μg/ml 2 to 3 months after surgery.

Figure 2 shows the effect of clip removal. There was a marked reduction in the blood pressure of 1K1C (Figure 2 A), but no statistically significant change was detected in the concentration of TRSS (Figure 2 B) after clip removal, which remained about two times higher than that of NC. Total plasma protein levels of 1K1C and NC were the same and did not change significantly after clip release (Figure 2 C), indicating that the specific activity of TRSS was constant for both groups.

The data in Figures 1 and 2 show that there is an increase in TRSS during the chronic phase of 1K1C hypertension and that unclipping, which normalized pressure, had no effect on the increased level of TRSS.

**Chromatography of Rat Plasma TRSS**

The chromatographic behavior of 1K1C and NC TRSS during desalting on AG11-A8 ion retardation resin was the same as that previously reported for human TRSS (Figure 1). The spasmogenic activity of both samples was eluted in the breakthrough region (40 to 50 ml), and the recovery of spasmogenic activity was 100% ± 20% for both plasma sources.

Figure 3 shows the elution diagrams obtained after equilibrium chromatography on SP-Sephadex for BK (Figure 3 A), desalted TRSS from NC (Figure 3 B), and 1K1C (Figure 3 C). Both samples of TRSS contained spasmogenic activity that was eluted in regions P₁ (4 to 10 ml) and P₂ (11 to 20 ml) as well as P₃ (35 to 47 ml). In five experiments (five to eight rats per group) the sum of the activity in regions P₁ + P₂ of 1K1C was two to six times larger than that for NC. The accurate measurement of spasmogenic activity in P₁ and P₂ was complicated by the presence of potentiating peptides. However, in separate semiquantitative experiments not documented here, the amount of BK potentiators in regions P₁ and P₂ of 1K1C and NC was found to be essentially the same.

The spasmogenic activity in region P₃ from 1K1C and NC plasma corresponded to BK in elution position and pharmacological properties (see below). The small shoulder on the ascending side of P₃ suggests that spasmogenic activity other than BK may have been eluted in this region of the effluent. No potentiating activity was detected in the effluent corresponding to P₃. A wide range of TRSS activity, 0.1 to 1.2 μg BK equivalents/ml plasma, was recovered in the P₃ region for both 1K1C and NC (five experiments, five to eight rats per group). The variation was too large to distinguish between 1K1C and NC plasma and much larger than that found for individual samples of human plasma submitted to the same chromatographic procedures.

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**Figure 1.** Changes in the levels of TRSS activity of one-kidney, one-clip hypertensive rats (1K1C) after clip implantation. A. Plethysmographic blood pressure. B. TRSS activity measured with the isolated guinea pig ileum and reported as bradykinin equivalents/ml plasma. Values are means ± SEM, n = 8. *p < 0.05 when compared to the previous measurement.

**Figure 2.** Effect of unclipping on direct mean arterial blood pressure (A), TRSS activity (B), and plasma protein (C) of 1K1C. The clip on the left renal artery was implanted 75 days before the acute experiment. A. •••••••• RH; •••••••• normotensive controls (NC). B and C. •••••••• RH; □ cannulated intact rat. TRSS activity was measured with the isolated guinea pig ileum and reported as bradykinin equivalents/ml plasma. The values are reported as means ± SEM, n = 7. *p < 0.05 when 1K1C and NC were compared for each time period.
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Figure 3. SP-Sephadex chromatography of TRSS. The column, 0.28 x 50 cm, was developed under equilibrium conditions with 0.02 M Tris-HCl buffer pH 7.7, containing 0.06 M NaCl at 6.0 ml/hr, 23° C (see reference 6). The samples applied to the column corresponded to 6.5 ml plasma treated with trypsin and desalted on AG11-A8 resin, as described in Methods. Spasmogenic activity in the effluent, measured directly with the isolated guinea pig ileum, is reported as bradykinin (BK) equivalents. The recovery of biological activity was 80% to 90%. A. Elution diagram of 25 μg of synthetic BK, which was recovered in 70% yield. B. Normotensive control (NC) rats. C. 1K1C. P = pool.

After separation from P₃, the P₁ and P₂ were combined and submitted to gel filtration on Sephadex G-25. Figure 4 shows the elution diagrams obtained for (P₁ + P₂) from 1K1C (Figure 4 B) and NC (Figure 4 C). Spasmogenic activity eluted in Regions I (70–100 ml) and II (101–112 ml) was present in TRSS from both groups, but 1K1C contained five to 15 times more activity in Region II than NC and about two times more activity in Region I. Potentiating peptides were detected in the entire effluent containing spasmogenic activity. Region II also corresponded to the elution position of BK (Figure 4 A), but this peptide had been removed by the previous SP-Sephadex chromatography step. Angiotensin II (AII), the octapeptide, was eluted after the nonapeptide BK. The elution position of aromatic amino acids and peptides containing aromatic amino acids from Sephadex G-25 was retarded with respect to the position expected on the basis of molecular weight, presumably because of adsorption effects. The possibility that the retardation was due to the ion-exchange properties of partially oxidized Sephadex was ruled out by the fact that AII was eluted in the same position when the solvent was 5% acetic acid or 5% acetic acid containing 0.2 M NaCl. Furthermore, the more basic nonapeptide BK, which contained phenylalanine rather than tyrosine, was eluted before AII.

Properties of Fractions P₁, P₂, and P₃

Fraction P₂, like BK, was inactivated by rabbit brain endo-oligopeptidase A, chymotrypsin and carboxypeptidase B and was insensitive to trypsin. Fraction P₁ could be distinguished from BK and P₂ by a two- to threefold activation by chymotrypsin, stability to inactivation by rabbit brain endo-oligopeptidase A, which hydrolyzes the Phe⁷-Ser⁸ peptide bond of BK and higher homologs, and the slower rate of inactivation by carboxypeptidase B. The P₁ was distinguished from BK and P₂ and was similar to P₂ on the basis of endo-oligopeptidase A stability and the slower rate of inactivation by carboxypeptidase B. In contrast to P₁, however, P₂ was inactivated by chymotrypsin.

Fractions P₁, P₂, and P₃, like BK, contracted the guinea pig ileum. This activity, which was potentiated by BPP₄, contracted the cat jejunum and ileum, as well as the rat uterus, and relaxed the rat duodenum.

Figure 4. Gel filtration on Sephadex G-25 of Pools 1 + 2 (see Figure 3) derived from 1K1C (B) and NC (C) rats. The column, 0.9 x 250 cm, was developed with 5% (vol/vol) acetic acid, pH 2.7 at 12 ml/hr, 23° C. The spasmogenic activity of the effluent after lyophilization was measured with the isolated guinea pig ileum and is reported as bradykinin (BK) equivalents. The recovery of biological activity was 70% for 1K1C and NC. A. Elution diagram of albumin, BK, NaCl, and angiotensin II (AII). B and C. The solid line corresponds to conductivity of the effluent.
but had no effect on the rat colon. The properties of Fractions I and II were the same as those of P1 and P2, respectively.

The effects of P1 and P2 on the blood pressure of conscious rats indicated that they were different from BK. Figure 2 shows that P1 could be distinguished from BK because it was hypertensive rather than hypotensive. Even though P2, like BK, decreased blood pressure, it could be distinguished from BK because it was not inactivated in the pulmonary circulation. As shown in Figure 5, an equidepressor dose of BK injected i.v. was 1000 ng, but when injected i.a. it was 25 ng, indicating 97.5% pulmonary inactivation, whereas the equidepressor dose of P2 was the same for both routes (Figure 2 C). Even if P2 also contained some potentiating peptides, we would not expect complete pulmonary protection because the lung has been found to contain kininases other than converting enzyme. On the basis of the hypotensive effect and pulmonary inactivation (not documented here), P3 corresponded to BK.

Discussion

The major findings reported here are that BK is not the only spasmodogenic substance released by extensive trypsin hydrolysis of NC plasma and that the levels of TRSS are elevated about twofold in 1K1C. In contrast, BK was the only spasmodogenic substance detected when equivalent amounts of human plasma were processed by the same chromatographic methods. The BK-potentiating peptides present in the TRSS of both species were separated by chromatography from BK but not from rat non-BK TRSS. Non-BK TRSS (P1 + P2) plus potentiating peptides accounted for 60% to 70% of the total TRSS in 1K1C plasma. Since the potentiating peptide activity present in Fractions P1 + P2 appeared to be the same in NC and 1K1C plasma, and the amounts of P1 + P2 + potentiating peptides in RH plasma were as much as two- to sixfold higher in RH than in NC plasma, it is suggested that P1 and P2 were responsible for the increased TRSS in 1K1C plasma.

Other investigators have also reported increased levels of kininogen in the plasma of 1K1C. In these experiments, kininogen was also determined by trypsin treatment and the spasmogonic activity measured with rat uterus and cat jejunum and/or guinea pig ileum. Recently, Corthorn and Croxatto attributed the increase in plasma kininogen to a four- to fivelfold increase in low molecular weight kininogen on the basis of the elution position from QAE-Sephadex and the measurement of TRSS with the rat uterus and cat jejunum. The observation by Werle et al. attributed rat kininogen levels increased 3.5-fold after extirpation of both kidneys was also based on extensive trypsin hydrolysis and measurement of spasmodogenic activity with the rat uterus. It is possible that these investigators observed a phenomenon similar to that described here, but no distinction could be made between BK and TRSS such as P1 and P2 because only bioassays were used, that is, without the chromatographic resolution of TRSS into BK and non-BK spasmodogenic substances.

Studies are underway in our laboratory to isolate and characterize P1 and P2. However, they can be distinguished from BK on the basis of the data presented here. Larger homologs of BK having lysine immediately before the BK moiety can be excluded by the fact that trypsin treatment of P1 and P2 did not increase the biological activity. Stability to trypsin and chymotrypsin, potentiation by BPPβ, relaxation of the rat duodenum, contraction of the cat jejunum and ileum, and absence of an effect on rat colon distinguish P1 and P2 from A1, AII, and tetradecapeptide. "Tryptensin," a vasopressor substance released from Fraction IV by trypsin and kallikrein at an acid pH, has been shown to be AII.

Earlier studies have indicated that the properties of P1 and P2 are not consistent with those of other biologically active substances present in plasma or in the trypsin-treated plasma of hypertensive animals. Spasmogenic substances with properties similar to those of P1 and P2 have also been detected in the TRSS from plasma of arthritic rats whose kininogen level was also two to three times higher than for normal rats. However, the question of similarity or identity among these substances can only be resolved after the structure of each is determined.

Two new biologically active peptides that are structurally related to BK have recently been described in trypsin hydrolysates of plasma. Okuda and Arakawa isolated [des-Pro3]-BK from human plasma Fraction IV-B, which potentiated the action of BK on the isolated uterus, but was about 1000 times less potent on the
same tissue. After this paper was submitted for publication, Okamota and Greenbaum described the isolation of T-kinin (Ile-Ser-BK) from the trypsin-treated plasma of normotensive rats. They have found that T-kinin is 1.3 and 2.5 times less active than BK on the rat uterus and guinea pig ileum, respectively. The potency of the depressor effect of T-kinin on rat blood pressure after i.v. injection was between that of Met-Lys-BK and BK, but no comparisons were made between i.v. and i.a. routes of injection. P had properties similar to those of T-kinin, but appeared to differ in what would be expected in terms of sensitivity to endo-oligopeptidase A and with respect to pulmonary inactivation. The relationship of P, to T-kinin will be studied in the future when direct comparisons can be made.

The present study has demonstrated that these new spasmonic substances in rat plasma are present. The study also shows that these substances are increased in 1K1C and do not decrease after unclipping. Even though the structures and relevance of Fractions P, and P, have not been demonstrated in the present study, it is clear that further study is needed to determine the biological significance of the data reported here for this model of hypertension and in experimental arthritis.

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