Role of the Pressor Action of Angiotensin II in Experimental Hypertension

By Donald T. Pale, Frederick D. Masucci, George S. Denning, Jr., Frank Sipos, and Dyral C. Fessler

ABSTRACT

Experiments with rabbit aortic strips and pithed rats indicated that L-Sar-8-Ala-angiotensin II (Sar-Arg-Val-Tyr-Val-His-Pro-Ala) is a specific competitive antagonist of the vascular action of angiotensin II. Norepinephrine-induced hypertension was not affected by infusions of L-Sar-8-Ala-angiotensin II which evoked a dose-related reduction of angiotensin II-induced hypertension in conscious rats. Infusions of L-Sar-8-Ala-angiotensin II that caused a dose-related reduction of the blood pressure of conscious rats in the acute phase of unilateral renal hypertension were ineffective during the chronic phase of such hypertension. Infusions of L-Sar-8-Ala-angiotensin II lasting 1 hour did not reduce the blood pressure of normotensive, spontaneously hypertensive, or metacorticoid hypertensive conscious rats. These data indicate that, under certain experimental conditions, the blood pressure of rats during the acute phase of unilateral renal hypertension is maintained by the endogenous angiotensin II previously demonstrated to be present in the plasma in supernormal concentrations. The blood pressure of normotensive, spontaneously hypertensive, metacorticoid hypertensive, and chronic unilateral renal hypertensive rats previously shown to have normal plasma levels of renin and angiotensin II appeared to be independent of the pressor action of endogenous angiotensin II. This is additional direct evidence implicating the pressor action of angiotensin II in the etiology of renal hypertension.

KEY WORDS specific competitive inhibition renal hypertension metacorticoid hypertension spontaneous hypertension peptide synthesis rabbit aortic strips pithed rats conscious rats

The role of angiotensin II in experimental hypertension in the rat has not been defined completely. Studies of plasma renin and angiotensin II concentrations in experimental renal hypertension and other hypertension models have yielded equivocal and often conflicting conclusions (1–3). Correlation of plasma concentrations of renin or angiotensin II with the level of arterial blood pressure in hypertension does not prove a cause and effect relationship. In fact, the rate of formation and metabolism of angiotensin II under various conditions is not known, nor is it clear whether modified pressor sensitivity to angiotensin II is a possible cofactor in hypertension syndromes (4).

A direct method of demonstrating a role for angiotensin II in experimental hypertension would be to use a specific antagonist of some component of the renin-angiotensin system. One aspect of this system which has received considerable attention during the last two decades is the interaction of angiotensin II with its vascular receptor sites. The structure of a commonly utilized synthetic analog of angiotensin II (Hypertensin, CIBA) is shown in Figure 1. Also shown is the structure of L-Sar-8-Ala-angiotensin II (Sar-Arg-Val-Tyr-Val-His-Pro-Ala), a compound with properties characteristic of a specific competitive antagonist of the vascular action of angiotensin II. This report briefly summarizes these properties and describes our experience with L-Sar-8-
Ala-angiotensin II in attempting to define a role for the pressor action of angiotensin II in several experimental hypertension models in the rat.

Methods

SYNTHESIS OF 1-SAR-8-ALA-ANGIOTENSIN II

T-butyloxy carbonyl (Boc)-alanine resin-ester (76 g, 0.79 mmol/g) was placed in a stirred reaction vessel (5). The resin was swelled in chloroform (analytical grade) for 20 minutes and thereafter washed with three 500-ml portions of glacial acetic acid (all wash operations were repeated three times for 5 minutes with each solvent). The Boc-protecting group was removed by in HCl in anhydrous acetic acid after 40 minutes. The resin then was washed with 500-ml portions of glacial acetic acid, absolute ethanol, chloroform, and methylene chloride, and a threefold excess (2.37 mmol) of Boc-proline was added in methylenechloride. The reaction mixture was stirred for 12 hours. Thereafter the whole operation, starting with the acetic acid wash step, was repeated for each amino acid, except for Boc-N^m-benzyl histidine and Boc-nitroarginine. In these two cases, a DMF-methylene chloride solvent mixture was used for the coupling step. After the coupling of Boc-O-benzyl tyrosine, some resin peptide was removed, and the synthesis was continued with 48.7 g (29 mmol) of resin-peptide. After the last coupling step, the resin-peptide was washed with DMF, ethanol, acetic acid, ethanol, and trifluoroacetic acid.

The resin-peptide (Z-sarcosyl-N^m-nitro-L-arginyl-L-valyl-O-Bzl-L-tyrosyl-L-valyl-N^m-Bzl-L-histidyl-L-prolyl-L-alanine resin-ester) was suspended in 500 ml of dry trifluoroacetic acid, and a stream of dry hydrogen bromide was passed rapidly into the suspension. After 40 minutes, the resin was filtered off and treated three more times with hydrogen bromide and trifluoroacetic acid for 40 minutes. The filtrates were concentrated in vacuo at 20°C to an oily residue which was solidified by trituration with absolute ether. The product was dried in a desiccator over potassium hydroxide. The overall yield of the protected octapeptide-hydrobromide was 25.7 g.

The protected peptide (sarcosyl-N^m-nitro-L-arginyl-L-valyl-L-tyrosyl-L-valyl-N^m-Bzl-L-histidyl-L-prolyl-L-alanine hydrobromide, 12.8 g) was dissolved in 350 ml of 50% aqueous acetic acid and hydrogenated over a mixture of Pd/BaSO_4 (58.2 g) and Pd/C (10%, 5.0 g) for 72 hours at atmospheric pressure. The mixture was filtered, diluted with water (400 ml), and lyophilized. The gummy solid was dissolved in water (40 ml) and precipitated by addition of acetone (1200 ml). The resulting precipitate was filtered, dissolved in water, and lyophilized to yield 6.32 g of crude peptide hydrobromide.

The crude product (sarcosyl-L-arginyl-L-valyl-L-tyrosyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-alanine hydrobromide, 12.4 g) was purified in 2-g batches by gel filtration on Sephadex G-25 in 1% acetic acid to produce 4.6 g of colorless, lyophilized peptide. The protected peptide (sarcosyl-L-arginyl-L-valyl-L-tyrosyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-alanine triacetate hydrate). The optical rotation was determined using a Perkin Elmer model 141 polarimeter: [α]_D^25 = -78.0° [c = 0.24, 1% acetic acid]. The isolated product contained two trace impurities detectable by thin layer chromatography using Merck silica gel HF-254 plates with Pauly spray reagent. In n-butanol/acetic acid/water (6:2:3, v/v) the product appeared at an R_f of 0.10 and a trace impurity was found with an R_f of 0.45. In n-butanol/acetic acid/water/pyridine (9:2:6:7, v/v) the product R_f was 0.35 with impurities at R_f 0.23 and R_f 0.65. The higher R_f impurity in the second solvent system was isolated by ion-exchange chromatography on Sephadex SE-C25 by elution with an ammonium acetate gradient (0.05-0.5 M) in 1% acetic acid. This component represented about 2% of the mixture and appeared by amino acid analysis to be a hexapeptide corresponding to the residue sequence 3-8 of the product peptide.
ANGIOTENSIN II AND EXPERIMENTAL HYPERTENSION

The octapeptide (10 mg) was dissolved in 6N HCl (3 ml) and heated under nitrogen in a sealed tube for 18 hours at 110°C. The analyses were performed on a Japan Electron Optics Laboratory amino acid analyzer model JLC-5AH. The following amino acid ratios were found: Ala 1.00, Pro 1.12, His 0.93, Val 1.98, Tyr 0.76, Arg 0.90, Sar 0.90.

Another sample of the peptide was hydrolyzed for 24 hours under the conditions described above. The amino acid mixture obtained was incubated with L-amino acid oxidase at 37°C and pH 7.2 for 48 hours. Amino acid analysis after this incubation indicated that 7.2% of the histidine in the product peptide was D-histidine. This is in agreement with the report of Jorgensen and Windridge (6) that the use of N-m-benzylhistidine results in some racemization of histidine.

RABBIT AORTIC STRIPS

Sections of rabbit thoracic aorta were obtained and prepared for experimentation as described previously (7). An equilibration period of 2 hours in Krebs-bicarbonate solution, an agonist (angiotensin II or norepinephrine, 0.1 ml) was added to the tissue bath at not less than 30-minute intervals. The antagonist (1-Sar-8-Ala-angiotensin II) was allowed 3 minutes of contact with the tissue before the addition of an agonist. Paired strips from the same rabbit were used; one received the antagonist, and the other served as a control. All pairs of strips were initially exposed to agonist in the absence of the antagonist to determine whether both strips possessed the same degree of sensitivity. The subsequent dose-response curves were evaluated in terms of percent of maximum contraction of the control strip. Concentrations of drugs are expressed as molarity of the free base in the bath medium.

PITTED RATS

Rats were pithed, cannulated, and prepared for experimentation as described previously (7). The antagonist, 1-Sar-8-Ala-angiotensin II, was infused through a cannula in the jugular vein at a rate of 0.004 ml/min. The infusion method was used, because preliminary experiments indicated that the biologically effective half-life of this peptide was short (approximately 12 minutes) and that the antagonism of angiotensin II-peptide was short (approximately 12 minutes). To test the effects of 1-Sar-8-Ala-angiotensin II on animals with normal blood pressures, after the recovery period and 2 hours of control blood pressure recording, a 1-hour infusion of saline or 1-Sar-8-Ala-angiotensin II was initiated. This was followed by another hour of blood pressure recording.

Unilateral renal hypertension was induced in a series of rats by constriction of the aorta between the origins of the two renal arteries in conjunction with ligation of the left ureter (8, 9). The 2-week period following coarctation of the aorta was designated the acute phase of unilateral renal hypertension and the period beyond 2 weeks was called the chronic phase (10). Thus, 6-7 days after the aortic stenosis operation, most of the animals were prepared for the recording of blood pressure and the infusion of saline or 1-Sar-8-Ala-angiotensin II. The remainder of the animals were prepared 4 to 5 weeks after the operation. The experimental protocol in both cases was identical to that described previously (7).

Spontaneously hypertensive male rats (250-300 g), originating as hypertensive mutants of a Wistar strain in Japan (11) and perpetuated by random inbreeding at the Animal Research Center of The Norwich Pharmacal Company, were prepared for the recording of blood pressure and the infusion of saline or 1-Sar-8-Ala-angiotensin II. Following the recovery period and 3 hours of control blood pressure recording, the experimental protocol for the preceding series of animals was followed exactly.

Metacorticoxid hypertension (12) was induced in Sprague-Dawley rats weighing 225 g. Deoxycorticosterone acetate was prepared as a 1%
angiotensin II did not significantly (P > 0.05) alter the norepinephrine dose-response curve. The potency of 1-Sar-8-Ala-angiotensin II as an antagonist of the contractile responses of rabbit aortic strips to angiotensin II was standardized by determining a pA2 value according to the method of Schild (13). The mean pA2 value obtained for 3 minutes of contact time was 8.61 ± 0.03 (n = 14).

No evidence was obtained in any of the in vitro studies to indicate the presence of intrinsic activity which could be attributed to 1-Sar-8-Ala-angiotensin II.

PITHED RATS

The intravenous infusion of 1-Sar-8-Ala-angiotensin II (1-10 µg/kg min⁻¹) caused a dose-dependent antagonism of angiotensin II-induced pressor effects in pithed rats. Figure 3 shows that partial dose-response curves for angiotensin II shifted to the right during infusions of 1-Sar-8-Ala-angiotensin II (1 µg/kg min⁻¹) while partial dose-response curves for phenylephrine, vasopressin, and tyramine were not significantly (P > 0.05) altered. Figure 3 also shows that the antagonism of angiotensin II-induced blood pressure responses was surmountable. Infusions of 1-Sar-8-Ala-angiotensin II at doses of 2-10 µg/kg min⁻¹ caused an antagonism of the angiotensin II-induced pressor responses which was of
FIGURE 4

Effect of l-Sar-8-Ala-angiotensin II infusions on the elevated blood pressure of conscious rats maintained by infusions of angiotensin II (a) or norepinephrine (b). The solid bars on the abscissas denote the interval of 1-Sar-8-Ala-angiotensin II infusions. In a: • = control, N = 13; o = 1-Sar-8-Ala-angiotensin II (1 μg/kg min⁻¹), N = 8; x = 1-Sar-8-Ala-angiotensin II (5 μg/kg min⁻¹), N = 8. In b: • = control, N = 7; x = 1-Sar-8-Ala-angiotensin II (5 μg/kg min⁻¹), N = 8. All values are means ± SE.

such a magnitude that it was technically impracticable to demonstrate the surmountable nature of the antagonism. Infusions at a dose of 1 μg/kg min⁻¹ caused an initial small (3-4 mm Hg) and transient (4-5 minutes) elevation of the base-line blood pressure in pithed rats. This was followed by a small (4 mm Hg) but significant (P < 0.05) depression of the base-line blood pressure below the preinfusion level. Termination of the infusion was not accompanied by any remarkable blood pressure effects other than an apparent return of the base-line blood pressure to the preinfusion level.

CONSCIOUS RATS

Figure 4a illustrates the time trend of blood pressure for conscious rats receiving intravenous infusions of angiotensin II at 2.5 μg/kg min⁻¹ and the effect of infusions of 1-Sar-8-Ala-angiotensin II at 1 and 5 μg/kg min⁻¹ during the angiotensin II infusions. The effects of 1-Sar-8-Ala-angiotensin II at 2.5 μg/kg min⁻¹ were approximately between those of the 1 and 5 μg/kg min⁻¹ infusions. It is apparent that the angiotensin II antagonism caused by 1-Sar-8-Ala-angiotensin II was dose-related. It is of interest that the blood pressure maintained by angiotensin II was not significantly (P > 0.05) reduced below the preangiotensin II blood pressure level even by the 5 μg/kg min⁻¹ infusion dose. Figure 4b shows the time trend of blood pressure for conscious rats receiving intravenous infusions of norepinephrine (5 μg/kg min⁻¹). Also shown is the negligible effect of infusions of 1-Sar-8-Ala-angiotensin II (5 μg/kg min⁻¹) on the blood pressure of these animals. None of the blood pressure values shown for the control animals was significantly (P > 0.05) different from those shown for the 1-Sar-8-Ala-angiotensin II-infused animals. Infusion of 1-Sar-8-Ala-angiotensin II at 10 μg/kg min⁻¹ in

FIGURE 5

Effect of 1-Sar-8-Ala-angiotensin II infusions on the blood pressure of conscious rats with acute (a) and chronic (b) unilateral renal hypertension. The solid bars on the abscissas denote the interval of saline or 1-Sar-8-Ala-angiotensin II infusions. In a: • = saline control, N = 8; o = 1-Sar-8-Ala-angiotensin II (1 μg/kg min⁻¹), N = 8; x = 1-Sar-8-Ala-angiotensin II (5 μg/kg min⁻¹), N = 8; * = 1-Sar-8-Ala-angiotensin II (10 μg/kg min⁻¹), N = 12. In b: • = saline control, N = 7; * = 1-Sar-8-Ala-angiotensin II (10 μg/kg min⁻¹), N = 7. All values are means ± SE.
Conscious rats with unilateral renal hypertension in the acute phase were infused with 1-Sar-8-Ala-angiotensin II at doses of 0.62, 1.0, 2.5, 5, and 10 μg/kg min⁻¹. Figure 5a shows the time trend of the blood pressure throughout the experimental period and also illustrates the effects of infusions of 1-Sar-8-Ala-angiotensin II at three dose levels. It is apparent that the blood pressure responses to infusions of 1-Sar-8-Ala-angiotensin II were dose-related. Rats in the chronic phase did not respond to infusions of 1-Sar-8-Ala-angiotensin II at 10 μg/kg min⁻¹ with a significant (P > 0.05) decrease in blood pressure (Fig. 5b). Infusions of 1-Sar-8-Ala-angiotensin II at 30 μg/kg min⁻¹ into four of these animals did not cause any greater change in blood pressure.

Conscious normotensive rats were totally unresponsive to infusions of 1-Sar-8-Ala-angiotensin II at 5 and 10 μg/kg min⁻³, as indicated in Table 1. This table also summarizes the time trends of blood pressure in conscious spontaneously hypertensive and metacorticoid hypertensive rats and shows the lack of a significant effect of infusions of 1-Sar-8-Ala-angiotensin II (10 μg/kg min⁻¹) on the blood pressure values for both of these hypertension models.

**Discussion**

The evaluation of 1-Sar-8-Ala-angiotensin II utilizing rabbit aortic strips indicated that it possessed an extremely high affinity for the angiotensin II receptor site of vascular smooth muscle. The compound exerted no detectable intrinsic activity in these tests and exhibited an antagonism to the action of angiotensin II which was surmountable and reasonably specific. Further evaluation of 1-Sar-8-Ala-angiotensin II in pithed rats demonstrated that the dose-response (pressor) curve for angiotensin II was shifted in a parallel manner, the antagonism of angiotensin II was surmountable as well as specific, and the apparent intrinsic activity was of a low order of magnitude. An extensive pharmacological
investigation of l-Asn-8-Ala-angiotensin II showed that it was a specific competitive antagonist of the action of exogenous and endogenous angiotensin II on vascular smooth muscle (7). The brief pharmacological profile presented for l-Sar-8-Ala-angiotensin II is similar to that of l-Asn-8-Ala-angiotensin II and indicated that both compounds are specific competitive antagonists of angiotensin II. The antagonistic potency of l-Sar-8-Ala-angiotensin II is approximately 60-fold greater in vitro and 50-fold greater in vivo than that of l-Asn-8-Ala-angiotensin II. The high potency of l-Sar-8-Ala-angiotensin II as an angiotensin II antagonist made it a good candidate with which to pursue further the experiments in conscious animals initiated with l-Asn-8-Ala-angiotensin II (7). The latter experiments were discontinued because of the excessive quantity of l-Asn-8-Ala-angiotensin II it would have been necessary to synthesize to fulfill the objective of the present study.

Studies with conscious rats were carried out in an attempt to demonstrate the specific competitive antagonist properties of l-Sar-8-Ala-angiotensin II under more physiological conditions. The blood pressure of conscious rats with norepinephrine-induced hypertension was not affected by doses of l-Sar-8-Ala-angiotensin II which evoked a dose-related reduction of angiotensin II-induced hypertension. These observations not only verified the specificity and dose-response relationship but also indicated that the antagonism of angiotensin II by l-Sar-8-Ala-angiotensin II was not a tachyphylactic phenomenon. Of additional interest is the observation that the blood pressure of rats with angiotensin II-induced hypertension was reduced to the normotensive level. Central vasomotor and direct vasoconstrictor contributions were important for angiotensin II-induced hypertension in anesthetized greyhounds (14). It is apparent that either l-Sar-8-Ala-angiotensin II completely blocked both sites of angiotensin II action or only blocked the vasoconstrictor action, the central vasomotor contribution being insignificant in the rat. It was not possible to distinguish between these alternatives with the available data.

The experiments in conscious rats with norepinephrine- or angiotensin II-induced hypertension suggested the possibility of utilizing l-Sar-8-Ala-angiotensin II as a diagnostic tool to elucidate the role of angiotensin II in normal and hypertensive blood pressure regulation. The observation that infusions of l-Sar-8-Ala-angiotensin II into conscious normotensive rats did not evoke a hypotensive response indicates that angiotensin II does not have a prominent role in normal blood pressure regulation. This conclusion contradicts those of Bing and Poulsen (15) and Worcel et al. (16) who injected (iv) antiangiotensin II plasma and observed a transient hypotension in anesthetized normal rats. Curiously, Bing and Poulsen noted that the blood pressure of conscious normotensive rats was not altered by the antiantiagiotensin II plasma, while Worcel et al. indicated that conscious normotensive rats responded to antiantiagiotensin II plasma in the same manner as did anesthetized normotensive rats. The results with l-Sar-8-Ala-angiotensin II in conscious normotensive rats are in agreement with the results of inhibition of the formation of angiotensin II by a phospholipid renin preinhibitor (17, 18) and the results of inhibition of the conversion of angiotensin I to angiotensin II by the peptidase, pyrrolidone carboxylic acid-Lys-Trp-Ala-Pro (19). Neither antagonist of angiotensin II formation caused a significant hypotensive response in conscious normotensive rats.

Other investigators have found renal ischemia to be more pronounced and renin production stimulated to a greater extent following aortic constriction than after unilateral renal artery constriction in rats (20). A vasopressor agent resembling angiotensin II was detected in the circulation of rats with aortic coarctation only for a period of 2 weeks after unilateral injury. As a result of those studies, it was suggested that the acute phase of unilateral renal hypertension in these animals was dependent on a renal humoral mechanism, while the chronic phase was
independent of a direct vasoconstrictive effect of a humoral pressor agent (10). The present studies on conscious rats with unilateral renal hypertension, in which the antagonism of endogenous angiotensin II by 1-Sar-8-Ala-angiotensin II caused a dose-related reduction of blood pressure during the acute phase but not the chronic phase, support this suggestion. The results of the present study on rats with acute unilateral renal hypertension are in agreement with those studies utilizing acute hypertensive rats with unilateral renal artery constriction and an intact contralateral kidney which demonstrated that an elevated blood pressure could be reduced by treatment with the pentapeptide, pyrrolidone carboxylic acid-Lys-Trp-Ala-Pro (19) or with a phospholipid renin preinhibitor (17, 18). Some studies of chronic hypertensive rats with unilateral renal artery constriction and an intact contralateral kidney indicated that treatment with a phospholipid renin preinhibitor (17, 18) or that induction of immunization to angiotensin II (21) could reduce the elevated blood pressure of the chronic state. However, the hypertension of similarly prepared chronic hypertensive rats was not responsive to treatment with the pentapeptide, pyrrolidone carboxylic acid-Lys-Trp-Ala-Pro (19) or to the short infusions of 1-Sar-8-Ala-angiotensin II used in the present study. The reason for the discrepancy between the results of the two independent angiotensin II immunization studies is not apparent. The possibility that more extended infusions of 1-Sar-8-Ala-angiotensin II would cause an effect in this type of chronic hypertensive rat model cannot be discounted.

Unilateral renal injury (following aortic or renal artery constriction) accompanied by contralateral nephrectomy is associated with normal levels of renin and angiotensin II in the plasma of hypertensive rats (1, 23). Rats with this type of hypertension were not responsive to treatment with the pentapeptide antagonist of the conversion of angiotensin I to angiotensin II (19), to passive immunization with renin antibodies (24), or to active immunization against angiotensin II (22). In view of the unanimous evidence cited for a lack of participation of the renin-angiotensin system in rats with hypertension due to unilateral renal injury and contralateral nephrectomy, it does not seem unreasonable to predict that a similar lack of effect may occur with short infusions of 1-Sar-8-Ala-angiotensin II; only further experience will verify this contention.

Infusions of 1-Sar-8-Ala-angiotensin II into conscious spontaneously or metacorticoid hypertensive rats did not evoke significant reductions of blood pressure. The results indicate that these two types of hypertension are not due to a pressor action of the normal or low plasma levels of renin and angiotensin II previously found in these animals (25-28). Hypertension would be theoretically possible in the presence of normal renin and angiotensin II levels if vascular reactivity to angiotensin II had been increased by sodium retention (29). However, it was noted that the blood pressure of metacorticoid hypertensive rats also failed to respond to immunization against angiotensin II (21). Similarly, the blood pressure of spontaneously hypertensive rats was not affected by the specific competitive inhibitor of angiotensin II, 4-Phe-8-Tyr-angiotensin II (30). The possibility that more extended infusions of 1-Sar-8-Ala-angiotensin II or 4-Phe-8-Tyr-angiotensin II would cause an effect in these models cannot be discounted.

Each of the renal, metacorticoid, and spontaneously hypertensive rat models has been referred to at one time or another as the experimental counterpart of essential hypertension in man. In spite of immense effort in the laboratory with these experimental hypertension models, the question of whether clinical essential hypertension has its etiologic basis in the kidney is still not resolved. Tests with specific antagonists to renin or angiotensin II in man could provide important information on the clinical syndrome of essential hypertension.

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